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360. REACTIONS TO HOT ATMOSPHERES OF JERSEY COWS IN MILK

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(With 3 Figures)

The effects of acute exposure to controlled hot atmospheres on the smaller domestic animals, fowls and some lower animals have been reported in previous papers from this laboratory (1–7).

A review of the literature did not reveal any reports, with the exception of that of Regan & Richardson (8), on the reactions of cattle subjected to accurately controlled atmospheric conditions. However, much valuable information concerning the general systemic reactions and grazing habits of cattle has been recorded by Rhoad (9–15), Bonsma (16–18), Seath & Miller (19–20) and many others.

METHODS

Atmospheres

Various combinations of dry-bulb temperatures from 85 to 110° F. and of absolute humidity from 6 to 16 gr. of moisture/cu.ft.* of dry air were used on different days to ascertain the comparative effects of these two atmospheric variables upon the reactions of dairy cows. Air movement remained relatively constant at a velocity of about 60 ft./min.

General conditions

Four grade Jersey cows, commencing about 3–4 weeks after calving, were admitted twice a week for 10 weeks to the air-conditioned room in which the required temperature and humidity had been produced. The animals remained in the room from 8.00 a.m. for 7 hr. or until the rectal temperature reached 107° F. Animals were tied so as to permit them to lie down or stand at will. Water at atmospheric temperature was offered on all occasions 3½ hr. after admission. At all other times the animals were stabled under external conditions. The experiment was carried out in the late autumn and early winter (April, May, June), during which time the dry-bulb temperature varied from 55 to 70° F. and absolute humidity from 2 to 6 gr./cu.ft.

The cows were fed and milked twice a day at 7.30 a.m. and 3.30 p.m. On experimental days this was just before and just after the period of exposure. The ration consisted of lucerne chaff and cereal concentrates (bran, pollard and maize meal)—as no animal concentrate was available—and was calculated to contain 21 lb. dry matter, 1·5 lb. crude protein and had a nutritive ratio of 6.

Blood samples were drawn as required from the jugular vein 15 min. before entry and 15 min. before removal.

* Throughout this and the subsequent paper, humidity is expressed in grains per cubic foot.

The following observations were made in the stalls under atmospheric conditions before commencing each period and at half-hourly intervals in the room thereafter: rectal temperature—by clinical thermometer; pulse rate—by manual palpation of the ventral coccygeal artery; respiratory rate—by observing movements of the flank. Respiratory volume and body weight were recorded on admission to the room and at hourly periods while under test. Respiratory volume was measured by means of a mask and meter and read over 15–30 sec. periods. Animals were weighed on a weigh-bridge sensitive to 50 g. Urine, faeces and saliva were collected manually and the evaporative loss determined from the balance sheet.

The milk was weighed and analysed for butterfat by the Babcock method (21). Specific gravity was measured by the Quevenne lactometer (22) and total solids calculated by the modified Sharp and Hart equation (Herrington (23)).

Blood was analysed for serum calcium, inorganic phosphate, blood sugar and red blood cells. Serum calcium was estimated by the method outlined by Cameron & White (24). Solid sodium fluoride was used as an anti-coagulant for blood required for the other tests. Blood sugar was estimated by the method of Somogyi (25,26), while inorganic phosphates were calculated as outlined by Green (27).

Results

Rectal temperature

The mean rectal temperature of the animals under atmospheric conditions was $101{\cdot}17 \pm 0{\cdot}67°$ F.* The rectal temperature of only one animal rose above 107° F. during the time of exposure and then only at a dry-bulb temperature of 110° F. and maximum humidity. An increase in humidity of 2 gr./cu.ft. at a dry-bulb temperature of 105° F. had a greater effect upon rectal temperature than an increase of 5° F. at a humidity of 12 gr./cu.ft. (see Fig. 1*a*). At the lower temperatures and humidities, after an initial small rise, an equilibrium was set up and maintained throughout the experimental period, whereas at the higher values the rises were greater and equilibrium was established more slowly.

The inversion of the relative effects of 105 and 110° F. seen in Fig. 1*a* and *b* may be ascribed in part to some degree of acclimatization, as the former represented the first test period and the latter the last period of the series.

Pulse rate

The mean ante-room pulse rate of the animals was $68{\cdot}1 \pm 9{\cdot}1$ beats/min. The effect of air temperature upon the pulse rate was slight and inconsistent but increase in humidity was accompanied by some increase in pulse rate.

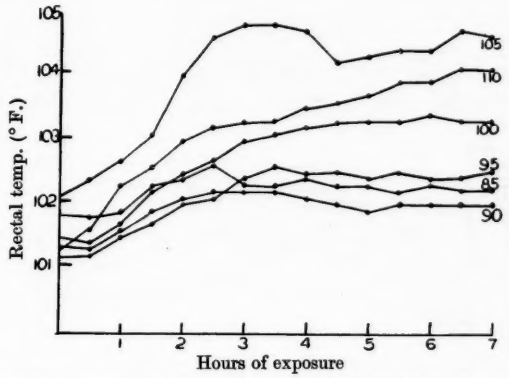
Respiratory rate

The mean ante-room respiratory rate was $25{\cdot}4 \pm 6{\cdot}1$ respirations per min. and this was observed to rise to about 160 respirations per min. at the higher combinations of temperature and humidity. Marked changes occurred in the rate and type of respiration with rise in both dry-bulb temperature and humidity, the latter again having the greater effect in the units given previously (Fig. 2*a* and *b*).

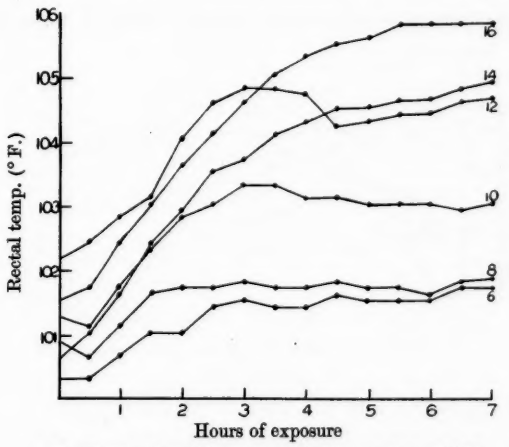
* Throughout this paper the standard deviation of the population is used as the measure of dispersion.

Equilibrium was attained under all conditions after a fairly rapid initial rise. Panting, i.e. open-mouth breathing, occurred when the respiratory rate approached 150 respirations per min.

When the respiratory rate and rectal temperature were compared it was evident that the rise in respiratory rate was largely antecedent to the rise in rectal temperature.



(*a*) Varying dry-bulb temperature. Absolute humidity 12 gr./cu.ft.



(*b*) Varying humidity. Dry-bulb temperature 105° F.

Fig. 1 *a*, *b*. The effect of (*a*) varying dry-bulb temperature (85–110° F.) at constant humidity (12 gr./cu.ft.) and (*b*) varying absolute humidity (6–16 gr./cu.ft.) at constant dry-bulb temperature (105° F.) on the rectal temperature of Jersey cows. Dry-bulb temperatures and absolute humidities are shown within the graphs.

Respiratory minute volume

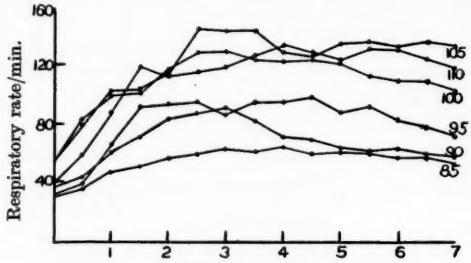
Although the measurement of respiratory volume was not as accurate as desired on account of the difficulty of fitting and holding the mask in position, there was some evidence that humidity was less important and that the minute volume increased with rise in dry-bulb temperature. The minute volume did not increase as rapidly as the rate, so that there was a decrease in tidal volume with temperature.

Evaporative weight loss

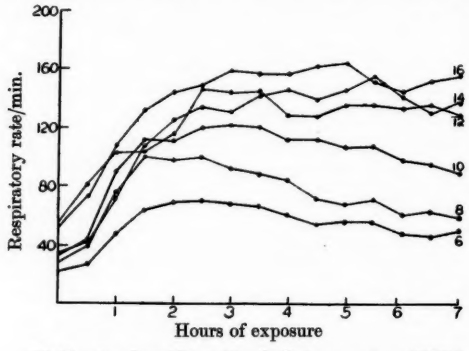
Evaporative weight loss appeared to be more affected by temperature than humidity in the units given (see Fig. 3). The observed evaporative loss (0·49–1·20 kg./hr.) was always much greater than that which could be accounted for by respiratory evaporation (36·2–67·7 g./hr.) even when possible discrepancies were taken into consideration.

Blood and milk

Only slight variations in the quantity of milk were observed on the day of the test, whereas on the following day a slight increase was almost invariably present.



(*a*) Varying dry-bulb temperature. Absolute humidity 12 gr./cu.ft.



(*b*) Varying humidity. Dry-bulb temperature 105° F.

Fig. 2*a*, *b*. The effect of (*a*) varying dry-bulb temperature (85–110° F.) at constant humidity (12 gr./cu.ft.) and (*b*) varying absolute humidity (6–16 gr./cu.ft.) at constant dry-bulb temperature (105° F.) on the respiratory rate of Jersey cows. Dry-bulb temperatures and absolute humidities are shown within the graphs.

The butterfat content, both percentage and total butterfat, of the afternoon milking showed slight variations but these were in no way related to the variations in temperature and/or humidity.

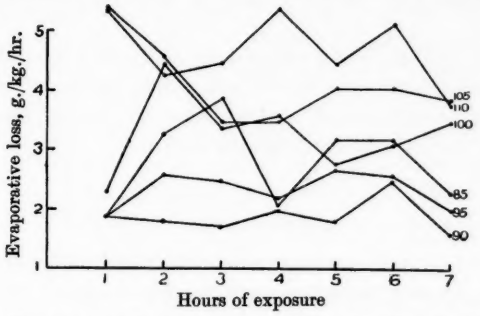
With rise in dry-bulb temperature at higher humidities, there was, at the beginning of the series, a marked rise in specific gravity from 1·0318 to 1·0346, and a rise in solids-not-fat from 10·05 to 10·72%. Smaller changes were observed in these properties towards the end of the series, due possibly to the effect of acclimatization.

Blood analyses showed that at high temperatures and humidities there was a marked

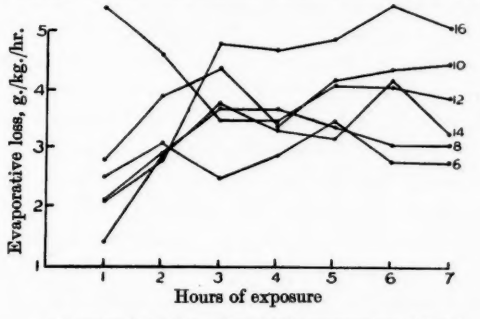
fall in inorganic phosphates to 2·95 mg./100 ml. blood from 4·96 ± 0·40 and a small drop in serum calcium to 8·64 mg./100 ml. serum from 10·58 ± 0·66. The decrease in blood sugar to 44·5 mg./100 ml. blood from a mean value of 55·22 ± 11·51 was in no way correlated with rise in temperature or humidity. The mean red blood cell count showed no significant variation from the mean value of 5·02 ± 0·24 million per cu.mm.

Behaviour

Some of the animals possessed minor peculiarities on entry to the room, even under 'normal' conditions. These characteristics, such as grinding the teeth and pawing the ground,



(*a*) Varying dry-bulb temperature. Absolute humidity 12 gr./cu.ft.



(*b*) Varying humidity. Dry-bulb temperature 105° F.

Fig. 3*a*,*b*. The effect of (*a*) varying dry-bulb temperature (85–110 ° F.) at constant humidity (12 gr./cu.ft.) and (*b*) varying absolute humidity (6–16 gr./cu.ft.) at constant dry-bulb temperature (105° F.) on the evaporative weight loss of Jersey cows. Dry-bulb temperatures and absolute humidities are shown within the graphs.

became accentuated with rise in dry-bulb temperature, especially at the higher humidities. Under the conditions of high temperature and humidity, when the rectal temperature was in the vicinity of 106° F., all animals showed signs of distress as evidenced by restlessness, stretching out of the head, protrusion of the tongue, licking and dripping of saliva from the mouth.

Panting was frequently observed at air temperatures of 105° F. and above, when the rectal temperature was above 104° F.

Dribbling of saliva from the mouth and mucus from the nostrils was not seen below a dry-bulb temperature of 100° F. or an absolute humidity of 10 gr./cu.ft. Individual cows

showed marked variation in the extent of dribbling which was seen when the rectal temperature varied from 103·1 to 104·8° F. At the higher humidities salivation became excessive and the loss approached 1 l./hr.

Rumination was observed only occasionally at air temperatures above 100° F. and then only for short periods of about 1 min. Air temperatures to 95° F. and rectal temperatures to 104° F. did not seem to have an appreciable effect on rumination.

At the lower temperatures water at atmospheric temperature was consistently consumed, but at the higher temperatures it was seldom taken by the animals. After the ingestion of water the rectal temperature dropped as much as 1·5° F. in 1–2 hr. and then commenced to rise again. The respiratory rate at the same time decreased by 30 to 50 respirations per min.

The motions of some animals became somewhat fluid under the more trying conditions when the rectal temperature was over 105° F., while under the worst conditions the motions developed a diarrhoeic consistency at a rectal temperature over 106·5° F. The motions, however, returned to 'normal' by the following day.

Discussion

The failure of the pulse rate to rise, and even a tendency to fall with increased environmental temperature has been reported by others (8, 20, 28). This suggests that increased thermal conductance in the superficial tissues through increased blood flow is not a major factor in the adaptation of these cattle to high temperatures.

The markedly increased respiratory effect strongly suggests to the casual observer that therein lies the chief method of adaptation; but comparison of the additional amount of water which could be evaporated in this way with the actual evaporative loss of the animal indicates that its importance is still secondary, that the increased pulmonary ventilation is a measure of the inadequacy of the quantitatively more important cutaneous evaporation to maintain a balance. Furthermore, the rate of evaporative loss remains fairly constant after the first hour, even when the respiratory rate continues to rise. On the other hand, it must be admitted that the rise in respiratory rate largely precedes the rise in body temperature. If the concept is accepted that the rate of percutaneous transudation (true insensible perspiration) is relatively constant, then the major part of the increasing evaporative loss with rising air temperature must be ascribed to sweat-gland activity. Sweat glands are fairly widely distributed in the hide but doubts have been expressed as to their functional capacity (8, 29). That they are relatively inefficient as compared with man, and that the absolute insufficiency increases with the heat stress, is fairly evident from the progressive rise in rectal temperature with increased air temperature.

Marked and important consequential effects of exposure to hot conditions are seen in alimentary function. Cessation of rumination, if long-continued, will of course affect the nutritional status. Field effects allied to this are reported by Seath & Miller (19). Refusal of water renders the thermal outlook even worse and reduces the circulatory efficiency. Increased water loss in the faeces must have similar consequences. Peripheral circulatory failure is less likely to occur in these cattle than in more efficient homeotherms with increased cutaneous blood flow and active sweating; but this immunity is bought at the expense of homeothermy. Salivation is a common mammalian heat reaction, but the cow

makes little use of it, as compared with cats and some other mammals which lick themselves very freely or with dogs which develop very rapid shallow breathing.

In these experiments nervous reactions were not very marked as the rectal temperature seldom reached the 107° F. level. That these 7 hr. exposures affected neither the milk production nor the total butterfat is noteworthy. The meaning of the fall in blood calcium and phosphate is not clear, unless it is associated with a respiratory alkalosis. (The acid-base balance has not been studied.)

In the matter of thermal tolerance, these milking Jersey cows occupy a somewhat intermediate position amongst mammals, being much more tolerant than rabbits (1) and pigs (2), rather more than cats (3), slightly less than dogs (4) and sheep (5), much less than man. The comparable reactions of calves and dry cows, and the effect of repeated and continuous exposure to hot atmospheres will be reported elsewhere.

Over a range of air temperatures 65–93° F. with 27–91 % relative humidity, Seath & Miller (20) found in the field that 1° F. change in air temperature caused 41–43 times as much change in respiration rate as 1 % change in relative humidity (i.e. about 0·1 gr./cu.ft.). In our tests, we found an increase of 20° F. to produce a rise of 80/min., and an increase of 10 gr./cu.ft. a rise of 100/min. This gives a ratio for 1° F./0·1 gr./cu.ft. of only 4. The more marked effect of humidity in our experiments is undoubtedly due to the higher ranges over which we worked.

SUMMARY

Four grade Jersey cows were exposed for 7 hr. twice a week to each of several atmospheres having dry-bulb temperatures ranging from 85 to 110° F. and absolute humidities ranging from 6 to 16 gr./cu.ft. The following reactions were studied: rectal temperature; pulse rate; respiratory rate; respiratory volume; evaporative loss; calcium, phosphate and erythrocyte composition of the blood; milk volume and butterfat content; behaviour.

Rectal temperature rose to higher values with less ready establishment of equilibrium the hotter the condition, but exceeded 107° F. only in the hottest atmosphere. (Dry-bulb temperature 110° F., absolute humidity 16 gr./cu.ft.)

Respiratory rate was similarly and markedly affected. In both cases, humidity had a marked effect as well as temperature, an increment of 0·4 gr./cu.ft. (approx. 4 %) in humidity having the same effect as 1° F. rise in air temperature. The highest average respiratory rate was 200/min. Respiratory minute volume rose less than the rate, so that tidal volume was reduced.

Pulse rate was essentially unaffected by a rise in temperature but tended to rise somewhat with humidity.

Evaporative loss was markedly increased by temperature, much less so by humidity. Increased pulmonary ventilation was inadequate to account for the observed losses, which are attributed to sweat-gland activity.

Behavioural changes included some licking, panting, salivation, mild agitation, cessation of rumination and refusal of water. Weakness and staggers did not occur under these conditions.

Neither milk nor butterfat production was essentially affected by the exposures. Blood calcium and phosphate levels fell, but the erythrocyte count was unchanged.

These findings provide basic data with which subsequent observations under varying conditions can be compared.

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361. REACTIONS OF JERSEY CALVES TO HOT ATMOSPHERES

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(With 3 Figures)

No reports were available which indicated the reactions of calves subjected to controlled atmospheric conditions. An investigation of this nature on adult dairy cows was reported from this laboratory (1).

Four grade Jersey calves, 8 weeks old, three female and one male, the progeny of the cows used in the previous investigation, were subjected to the same combinations of dry-bulb temperature and humidity. The experiment, extending over a period of 9 weeks, was undertaken in the winter months (May, June and July), during which the external dry-bulb temperature varied from 57 to 70° F. and the absolute humidity from 2 to 6 gr./cu.ft. General management practices were similar to those described in the former report.

The calves were fed twice a day with about $\frac{3}{4}$ gal. milk each. When the animals were not in the room, lucerne chaff, and a concentrate mixture of bran, pollard and maize meal were allowed *ad lib.* In regard to the observations the only difference from methods previously used was that both pulse rate and respiratory rate were recorded by means of a stethoscope. Blood samples were drawn and analysed as described previously.

Results

Rectal temperature

The mean rectal temperature of the animals under normal external atmospheric conditions was $101{\cdot}61 \pm 0{\cdot}69°$ F.* Rectal temperature was observed to increase with rise both in dry-bulb temperature and in humidity (see Fig. 1*a* and *b*). At constant humidity we observed that equilibrium was attained only at the lower temperatures 85–95° F. At a dry-bulb temperature of 110° F. and humidity of 16 gr./cu.ft., three of the animals were removed by the fourth hour, while the other animal remained in the room for the 7 hr. Even at a dry-bulb temperature of 105° F., when the humidity was 14 and 16 gr./cu.ft., two of the calves had to be removed at the sixth and fourth hours respectively.

Pulse rate

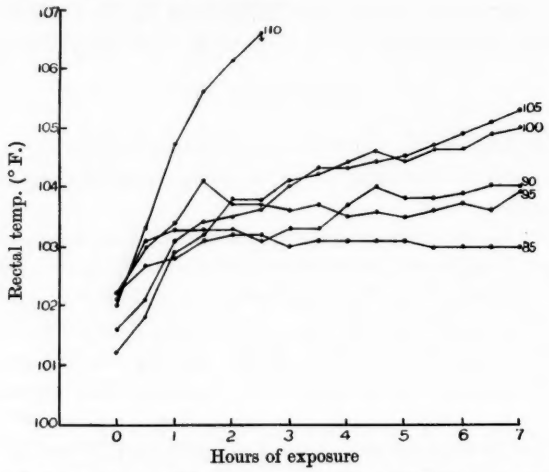
The mean ante-room pulse rate was $89{\cdot}8 \pm 11{\cdot}7$/min. The rate did not vary greatly with either dry-bulb temperature or humidity.

Respiratory rate

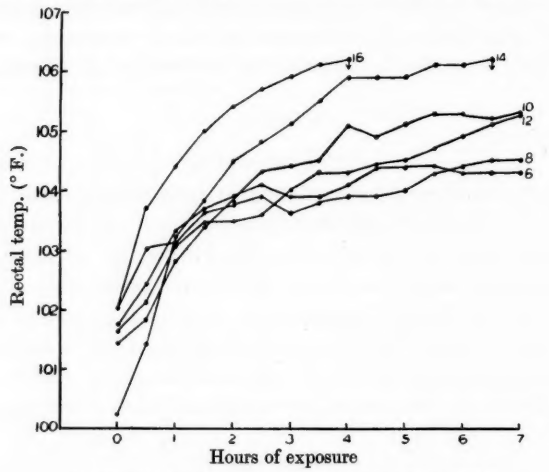
The mean ante-room respiratory rate was $21{\cdot}2 \pm 4{\cdot}4$/min. The respiratory rate was characterized in heat by an extremely rapid initial rise to a stable plateau (see Fig. 2*a* and *b*). The maximum rate observed was 256, approximately twelve times the ante-room

* Throughout this paper the standard deviation of the population is used as the measure of dispersion.

reading. The rate increased with increase both in absolute humidity and in dry-bulb temperature. At the lower temperatures, however, the rate showed some tendency to fall towards the end of the experimental period. Panting was frequently observed and was noted to occur at a rectal temperature which varied between 103·2 and 105·4° F., and when the respiratory rate was about 200/min.



(*a*) Varying dry-bulb temperature. Absolute humidity 12 gr./cu.ft.



(*b*) Varying humidity. Dry-bulb temperature 105° F.

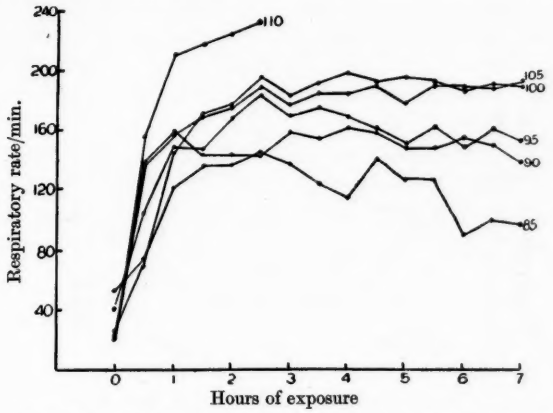
Fig. 1 *a*, *b*. The effect of (*a*) varying dry-bulb temperature (85–110° F.) at constant humidity (12 gr./cu.ft.) and (*b*) varying absolute humidity (6–16 gr./cu.ft.) at constant dry-bulb temperature (105° F.) on the rectal temperature of Jersey calves. Dry-bulb temperatures and absolute humidities are shown within the graphs.

Respiratory minute volume

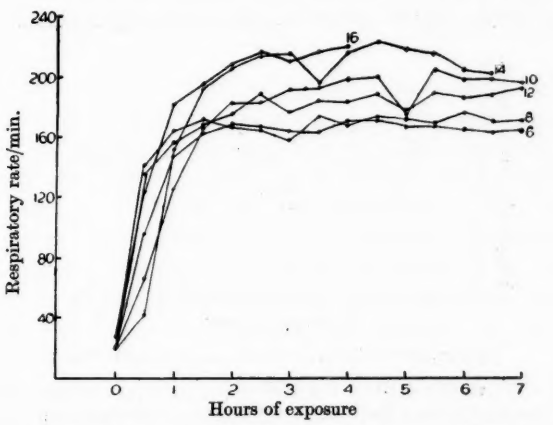
The difficulty of preventing leakage from the mask was encountered again in the calves, but examination of the records showed that there was a general tendency for an increase in respiratory minute volume with rise in dry-bulb temperature when the humidity was

held constant. This tendency was less evident when we examined the volume at varying humidity and constant dry-bulb temperature and was highest at the intermediate humidities.

Tidal volume did not vary considerably, except at the higher temperature and humidity when it decreased by about one-third.



(*a*) Varying dry-bulb temperature. Absolute humidity 12 gr./cu.ft.



(*b*) Varying humidity. Dry-bulb temperature 105° F.

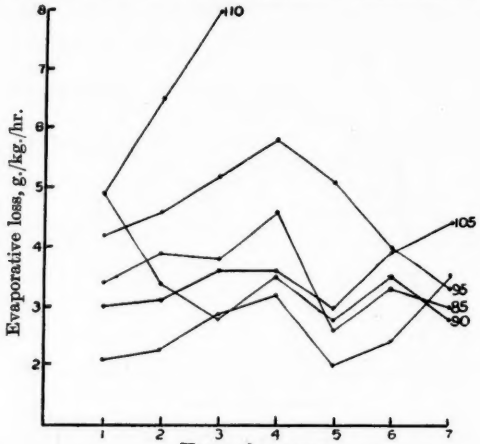
Fig. 2*a*,*b*. The effect of (*a*) varying dry-bulb temperature (85–110° F.) at constant humidity (12½ gr./cu.ft.) and (*b*) varying absolute humidity (6–16 gr./cu.ft.) at constant dry-bulb temperature (105° F.) on the respiratory rate of Jersey calves. Dry-bulb temperatures and absolute humidities are shown within the graphs.

Evaporative weight loss

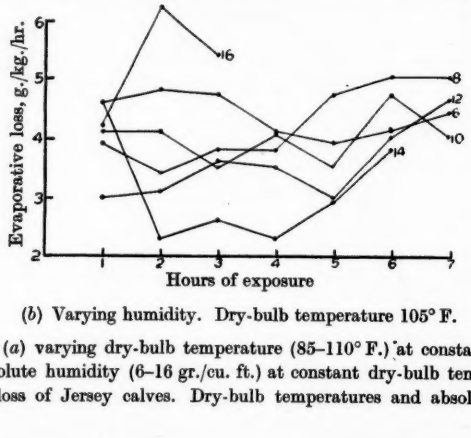
The loss did not appear to be affected by changes in absolute humidity but was higher at the higher dry-bulb temperatures (see Fig. 3). The observed evaporative loss (125–475 g./hr.) was always greater than that which could be accounted for by respiratory evaporation (78–63 g./hr.) even when possible discrepancies were taken into consideration.

Blood analyses

Blood analysis showed only small changes in both serum calcium and inorganic phosphates from the mean values of $10{\cdot}69 \pm 0{\cdot}86$ mg./100 ml. serum and $7{\cdot}23 \pm 0{\cdot}62$ mg./100 ml. blood respectively. Variation in the blood sugar was observed in the ante-room samples



(*a*) Varying dry-bulb temperature. Absolute humidity 12 gr./cu.ft.



(*b*) Varying humidity. Dry-bulb temperature 105° F.

Fig. 3*a*, *b*. The effect of (*a*) varying dry-bulb temperature (85–110° F.) at constant humidity (12 gr./cu.ft.) and (*b*) varying absolute humidity (6–16 gr./cu. ft.) at constant dry-bulb temperature (105 ° F.) on the evaporative weight loss of Jersey calves. Dry-bulb temperatures and absolute humidities are shown within the graphs.

but once again afternoon samples did not show any appreciable difference from the mean of $79{\cdot}70 \pm 21{\cdot}63$ mg./100 ml. blood. Red blood cell counts remained comparatively constant at the mean ante-room value of $8{\cdot}15 \pm 0{\cdot}31$ million/cu.mm.

Behaviour

Characteristics of the calves such as grinding of the teeth, pawing and licking, closely followed those of their respective dams and were accentuated on increase in severity of the conditions. Symptoms of distress were similar to those reported in the adult dairy cows.

Panting was observed to occur when the rectal temperature varied between 103·2 and 105·4° F.

Dribbling of saliva from the mouth was observed at air temperatures of 100° F. and above when the humidity was greater than 12 gr./cu.ft. Salivation commenced at a rectal temperature which varied between 103·6 and 106·2° F. and the maximum loss was 350 ml./hr.

Rumination seldom occurred when the air temperature was above 100° F. or when the rectal temperature varied between 104·8–106·2° F.

Water was rarely consumed by the animals and then only in small amounts of less than 2 l.

The motions became very soft and of a diarrhoeic consistency when the rectal temperature was in the vicinity of 106·6° F., which occurred only under the severe conditions.

Weakness of the hind limbs was observed in two of the animals when the rectal temperature was between 106·0 and 106·8° F.

DISCUSSION

The body temperature response of the calves to hot conditions varies markedly from those of the cows in three respects—more rapid initial rise; higher equilibrium values, especially under less severe conditions; and reduced effect of alterations in absolute humidity. These differences are even more striking in the response of the respiratory rate.

Calves, therefore, may be said to suffer a greater strain than cows in response to a given heat stress. This is substantiated by the greater necessity for their removal from the more severe atmospheres.

On the other hand, there are no marked differences between the rates of evaporative weight loss per unit body weight. In terms of unit surface area, however, the evaporative loss would be less for the calves than for the cows, suggesting a lowered efficiency of sweat glands, especially as the density of glands per unit area might be expected to be greater in the smaller and younger animal. The relatively smaller effect of humidity upon the calf reactions would not be inconsistent with this. It is also possible, however, that there is a smaller thermal conductance through the superficial tissues of the calf. The relatively greater discrepancy between cow and calf at lower temperatures would be consistent with this concept. It is evident that careful studies in partitional calorimetry are called for.

SUMMARY

Four grade Jersey calves were exposed for 7 hr. twice a week to each of several atmospheres having dry-bulb temperatures ranging from 85 to 110° F. and absolute humidities ranging from 6 to 16 gr./cu.ft. The following reactions were studied: rectal temperature; pulse rate; respiratory rate; respiratory volume; evaporative loss; calcium, phosphate, sugar and erythrocyte composition of the blood; behaviour.

Rectal temperatures rose rapidly to a higher level than was shown by cows under similar conditions, but maintained a steady equilibrium thereafter, except under the most severe conditions.

Respiratory rate responses resembled those of rectal temperature, the difference from those of cows being even more striking.

Humidity had relatively less effect upon the rectal temperature and respiratory rate responses of calves than of cows.

Pulse rate and tidal respiratory volumes were relatively unaffected, but minute respiratory volumes rose.

Evaporative loss per unit body weight resembled that of cows, except that humidity again had less effect. Pulmonary ventilation was inadequate to account for the observed losses.

Behavioural changes resembled those of the cows but weakness of the hind limbs was observed at rectal temperatures above 106° F. Blood calcium, phosphate, sugar and erythrocyte levels were not essentially affected.

Possible explanations for the lower thermal tolerance of calves, as compared with cows, are considered.

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362. STUDIES IN THE BACTERIOLOGY OF MILK

I. THE STREPTOCOCCI OF MILK

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The bacteria that occur in milk have frequently been studied in the mass by making counts of the organisms or by measuring their total chemical activity. Comparatively little attention has been given, however, to the composition of the bacterial populations in different types of milk. It is generally accepted that streptococci are frequently the dominant organisms, and much information is available concerning the coliform group; but for other bacteria and the individual species of streptococci there are few records of the frequency with which they occur. The question whether market milk possesses a special and characteristic microflora, or one that is too variable and heterogeneous to define, cannot be answered by the information at present available. The solution of practical problems in the dairy industry is often impeded by this lack of knowledge; too frequently the only approach must be an empirical one.

The object of the present investigation was to provide a clearer picture of the microbic populations in raw and pasteurized milks of varying purity. The general plan of the work was to make quantitative platings and to investigate the organisms that formed colonies. The pure cultures were studied for the purposes of identifying them and of discovering their properties, especially those properties that appeared to be of significance in relation to dairy products. Problems arising during the course of the diagnostic work were investigated with the object of improving methods of recognizing the more frequently occurring organisms.

This, the first paper of a series, describes work on the streptococci of milk. Subsequent papers will be concerned with the other major groups of bacteria and with the quantitative relationships of the organisms in different classes of milk.

Method of examining milk

In selecting milk for examination the intention was to secure specimens varying widely in bacteriological condition. Thus, three main types of raw milk were used: (1) samples obtained by aseptic milking, (2) milk produced under good or fairly good conditions and bottled on the farm, and (3) bulked milk in which heavily contaminated supplies were invariably represented.

Samples were examined in the raw state and also after pasteurization in the laboratory at 63° C. for 30 min. They were plated in duplicate on meat infusion agar containing 1% tryptone peptone and 0·25% glucose. Each plate inoculated with less than 1 ml. of the sample received an addition of 0·5 ml. of sterile skim milk. Plates were incubated for 5–6 days at 30° C. This combination of medium and incubation was expected to permit the growth of most types of the bacteria occurring in the samples. If this expectation was in fact realized, only the predominant organisms would be obtained from samples giving

high plate counts. No systematic attempt was made to detect minorities by selective culture. After incubation every colony on a suitable plate, or on duplicate plates, or on opposite sectors of a plate, was examined. An attempt was made to secure a sample consisting of 40–50 colonies. Each colony was transferred to a tube of litmus milk containing 0·25% yeastrel (a commercial yeast autolysate) and 0·25% glucose, and the cultures were incubated at 30° C. In some of the later work, in which milk was examined only after pasteurization, three modifications of the method were introduced: (1) the meat infusion of the plating medium was replaced by 0·5% meat extract, (2) an additional plate of each dilution was incubated at 37° C. for 2–3 days, and (3) colonies were transferred to plain litmus milk which was incubated at the same temperature as that used for the plate.

The first step in the identification of the organisms was to make transfers from the initial milk cultures to slopes of agar containing 0·5% each of meat extract, peptone and milk and 0·25% glucose. The resulting cultures were examined for morphology by Gram's method and for catalase. Further tests, considered to be appropriate to the organism in question, were then applied to the original cultures. It may be noted here that in the recognition of milk bacteria their morphological characters must be used with caution; certain streptococci when growing on agar produce an abundance of elongate cells, and in stained preparations certain corynebacteria and slime-forming rods may simulate streptococci or micrococci.

The study of streptococcal cultures

At the outset of this work it was decided to employ for the identification of streptococci methods that have been developed more especially by Sherman and his collaborators (1). There have been comparatively few independent reports on the application to the species that occur in milk of the differential criteria recommended by Sherman, and several workers (2, 3, 4, 5) have experienced difficulties in the use of some of the tests. Our findings in the examination of a long series of cultures provide a considerable volume of evidence concerning several of the methods employed. The details will be given later, but at this point it may be stated that the characterization of species suggested by Sherman has proved to be an invaluable basis for the quick identification of homofermentative strains. The biggest obstacle encountered in the study of streptococcal cultures was the lack of an easy and certain method of recognizing heterofermentative types. This difficulty was overcome when a cultural test for gas formation by such organisms had been devised (Gibson & Abd-El-Malek (6)).

The separation of the main groups of streptococci

The procedure adopted when an organism had been shown by microscopical examination and a negative result in the catalase test to be a streptococcus was to discover the main group to which it belonged. For this purpose tests for the following six properties have proved to be especially suitable.

(1) *Hydrolysis of arginine.* The method suggested by Niven, Smiley & Sherman (7) was used except that the medium was modified, 0·5% yeast extract being replaced by 0·25% of the yeast autolysate, yeastrel.

In the earlier stages of the work tests were made for the formation of ammonia in 4% peptone, but after the appearance of the paper by Niven *et al.* (7), the arginine medium, which gives identical but clearer results, was adopted.

(2) *Growth at* 45° C. Tests were made in litmus milk (free from spores of thermophiles) incubated in a water-bath. Litmus milk containing glucose (0·25%) and yeastrel (0·25%) was used when testing organisms that show little activity in plain milk.

(3) *Growth at* 8–12° C. Temperatures between these limits were obtained in a basement. They appear to be an adequate substitute for 10° C., a temperature that other workers have used. Tubes of litmus milk (containing glucose and yeastrel when testing certain organisms) were inoculated, chilled in cold water, and then placed at 8–12° C. If no change was detected within 2–3 weeks the result was interpreted as negative.

(4) *Survival at* 63° C. *for* 30 *min.* Resistance to heat is a useful differential character among the streptococci provided an adequate technique is used for its detection. Several factors are known to influence the result of a test of this property. Of special importance for the purpose of differentiation is the form of the test. Experience confirms the theoretical deduction that unless the organisms to be distinguished differ rather widely in the level of their resistance, the outcome of a test that depends on the complete sterilization of a bacterial suspension will show much variation. A carefully standardized quantitative method is too time-consuming for routine identifications, and the following procedure, which affords results of sufficient uniformity for the end in view, was used throughout this work.

In a sterile $\frac{5}{8}$ in. tube are placed 0·2 ml. of a fresh, moderately turbid lactose broth culture and then 5 ml. of skim milk (sterilized by intermittent steaming). Care is taken to avoid contaminating the side of the tube. The tube is placed in a water-bath at 63° C. with the milk level well below the water level, and is heated for 30 min. after the temperature of the milk has risen to 63° C. The tube is then cooled and a plate of glucose agar (the medium used in plating the milk samples) is poured with 0·2 ml. of the milk. The plate is incubated at 30° C. and is examined for growth after 3–4 days.

For every bacterium there is certain to be some heat treatment which permits the survival of a small proportion of cells, so that a few colonies will be produced in certain trials and none in others. Instances have been noted, for example, when *Str. lactis* was heated at 60° C. and when *Str. kefir* was heated at 63° C. The choice for differential purposes of 63° C. was made chiefly because this temperature is employed in the pasteurization of milk. An additional consideration is that resistance at 60° C., which has been more commonly used for specifying the thermoduric streptococci, is less clearly linked with the easily determined and valuable diagnostic character, the ability to grow at 45° C. Milk was adopted as the medium in which to heat the organisms partly because of the relative constancy of its composition and partly on account of the application of the results to dairy practice.

(5) *Action on litmus milk.* The main feature to receive attention was the reduction of the litmus. When it began before the indicator became pink, and was complete beneath the surface layer before the milk clotted, it was considered to be of the vigorous type. While this reaction is not entirely dependable, especially in the case of strains that have been cultivated artificially for long periods, it is useful for the detection of freshly isolated cultures of *Str. lactis*, *Str. cremoris* and many enterococci.

(6) *The formation of* CO_2 *from glucose.* A test for this property permits the recognition of heterofermentative streptococci. The technique has been described previously (6).

In the early stages of the work every culture was examined for its ability to tolerate

methylene blue at a concentration of 0·1 % in milk, but this practice was discontinued as the methods already detailed appeared to be adequate for the primary grouping.

Table 1 shows how the organisms were placed in five main groups by means of the tests just described. Frequently, among the streptococci isolated from a sample there would be several representatives of a single group, and in such an event the tests of heat resistance and gas formation were often applied only to some of the cultures. They were omitted entirely in cases where they were obviously superfluous, especially in the later stages of the work when the reliability of the other tests had been established.

Table 1. *The primary grouping of streptococci*

	Mastitis group	*Str. bovis*, *Str. thermophilus*	*Str. lactis*, *Str. cremoris*	*Str. faecalis*	Hetero-fermentative
Arginine hydrolysis	+	−	±	+	−
Growth at:					
45° C.	−	+	−	+	−
8–12° C.	−	−	+	+	+
Survival at 63° C. 30 min.	−	+	−	+	±
Vigorous reduction of litmus	−	−	+	±	−
CO_2 from glucose	−	−	−	−	+

Occasional exceptions to the reactions described in Table 1 may occur, but they do not detract greatly from the value of the tests for showing quickly the group to which an organism belongs. Anomalies become evident in any case by some irregularity in the results. All the atypical reactions that have been encountered are described later in this paper. Only one streptococcus for which the differentiation provided by Table 1 is not adapted has been isolated from milk during the course of the work. The organism resembled *Str. acidominimus* but differed from that species in some of its features.

Further investigations of streptococcal cultures

The cultures having been assigned to one of the main groups, confirmatory and differential tests that appeared to be suitable were made. In addition, representative strains, after having been replated to ensure purity, were subjected to a more detailed study. Among the strains selected for this purpose an effort was made to include any that did not appear to be typical of a recognized species. The subsequent discussions deal with the results obtained.

The methods employed are not new in the study of streptococci, but certain details will be mentioned so that the findings may be compared with other published data. If an incubation temperature is not specifically mentioned it was either 30 or 37° C., whichever was the more suitable for the organism being examined.

Inhibition by 0·1 % *methylene blue.* Skim milk, sterilized in bulk, and 1 % medicinal methylene blue in distilled water, sterilized in screw-capped bottles, were mixed and distributed in tubes with aseptic precautions. The tubes were inoculated immediately and were stoppered in order to prevent evaporation.

Inhibition by sodium chloride. The medium contained 0·5 % each of lactose, tryptone and meat extract and the chosen *W*/*V* percentage of salt. The salt and the other ingredients, both in concentrated solution, were sterilized separately in screw-capped bottles. Immediately before use measured volumes were combined and tubed aseptically. Tubes were inoculated with a loopful of a 24 hr. lactose broth culture and were incubated for

48 hr. In some of the work, agar of the same composition as the broth and prepared in the same way was used in poured plates.

Inhibition by 40% *bile.* The medium consisted of 4 parts of ox bile and 6 parts of a broth containing 0·5% each of lactose, peptone, tryptone and meat extract.

Haemolysis. Poured plates of 5% horse-blood agar were incubated for 48 hr. and were then placed in the refrigerator overnight. Ox-blood agar was used in a routine test for mastitis organisms.

Diastase production. Poured plates of 0·2% starch agar were incubated for 5 days at 30° C. and then flooded with iodine solution.

Aesculin and hippurate. Hydrolysis was determined by addition of ferric chloride after incubation for 5–7 days in a medium containing 0·1% aesculin, 1% sodium hippurate and 0·5% each of peptone, tryptone, meat extract and yeast extract. The two test substances were also used separately but there appeared to be no advantage in doing so. Strains that failed to grow were retested with 0·25% glucose in the medium.

All the types of streptococcus isolated were tested for action on casein (in milk agar or a casein agar) and on gelatin. *Str. liquefaciens* attacked both, but in all other trials the results were negative. The organisms were also examined for lipase production using an agar containing butterfat, cotton-seed oil or olive oil and testing with $CuSO_4$, but the findings were uniformly negative.

THE MASTITIS GROUP

For the purpose of this study the mastitis streptococci were treated as a unit, and the group diagnosis was merely supplemented by certain confirmatory tests. A character determined for all strains was their action on 5% ox-blood agar. The deep colonies were shown to be either weakly haemolytic or non-haemolytic. A proportion of the strains was also examined more fully and was found to possess the characters of either *Str. agalactiae* or *Str. dysgalactiae.*

THE *STR. BOVIS–STR. THERMOPHILUS* GROUP

In our experience organisms of this group are commonly the predominant streptococci in ordinary bulked milk immediately after pasteurization. The examination of samples of such milk thus provided abundant material for study. Every culture identified according to Table 1 was tested further in lactose broth and 0·1% methylene blue milk. Typical strains fermented lactose and showed no action on methylene blue milk. In litmus milk they formed acid and usually a curd; the reduction of litmus, if it occurred, was delayed until after the clotting and it rarely approached completion. These characters, in conjunction with those shown in Table 1, were expected to be sufficient to exclude other species of *Streptococcus.*

In the preliminary grouping of streptococci a considerable number of cultures were encountered which did not conform exactly to *Str. bovis* or *Str. thermophilus* as described by Sherman (1), but they seemed nevertheless to be closely related to these species. Some are capable of multiplying to a limited extent at temperatures close to 10° C. and also in milk +0·1% methylene blue. The organisms thus showed some resemblance to the enterococci and their identity was not obvious. Other problems arose when attempts were made to separate *Str. bovis* and *Str. thermophilus.* In the expectation that the latter could be distinguished by the ability to grow at 50° C. and by inactivity towards maltose,

tests for these properties were made but the results were often irregular. Salicin and aesculin were then introduced as differential test substances, and still other apparent anomalies came to light.

These findings prompted a more detailed study of the organisms. A collection of 173 cultures, containing representatives of the variations already noted, was assembled for this purpose. Characters common to all the cultures are shown at the foot of Table 2. They indicate the improbability that any of the organisms belong to species other than *Str. bovis* or *Str. thermophilus*. The properties in which the organisms differ are shown in the same table. In order to bring out the distribution of the variations the cultures have

Table 2. *Characters of* Str. bovis *and* Str. thermophilus

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
No. of cultures	14	10	8	3	22	15	9	11	12	9	7	18	27	8
Survival at 65° C. 30 min.	−	−	−	−	−	−	−	−	+	+	+	+	+	+
Growth at:														
50° C.	−	−	−	−	−	−	− or slight	− or slight	+	+	− or slight	−	−	−
16° C.	−	−	−	−	Very slow	+	Slow	Slow	−	−	−	−	−	−
10–13° C.	−	−	−	−	−	Slow	Very slow	Very slow	−	−	−	−	−	−
Growth in presence of:														
2% NaCl	+	+	+	+	+	+	+	+	−	Slight	+	+	+	+
3% NaCl	+	+	+	+	+	+	+	+	−	−	−	−	−	−
4% NaCl	−	−	−	−	−	Slight	Slight	Slight	−	−	−	−	−	−
0·1% methylene blue	−	−	−	−	−	−	Very slow	Very slow	−	−	−	−	−	−
40% bile	+	+	+	+	+	+	+	+	−	−	−	−	−	−
Aesculin hydrolysis	+	+	−	−	−	−	−	−	−	−	−	−	−	−
Diastase production	+	−	−	+	+	+	+	+	−	−	−	−	−	−
Acid production from:														
Maltose	+	+	+	+	+	+	+	+	−	Late	Late	Late	−	−
Raffinose	+	+	+	+	+	+	+	+	−	−	+	−	−	+
Inulin	−	−	−	+	+	+	−	+	−	−	−	−	−	−
Mannitol	−	−	−	−	−	+	−	−	−	−	−	−	−	−
Sorbitol	−	−	−	+	−	Late	Late	−	−	−	−	−	−	−
Salicin	+	+	+	−	+	+	+	+	−	−	−	−	−	−
Preferential utilization of sucrose and lactose	−	−	−	−	−	−	−	−	+	+	+	+	+	+
Blood agar	Inert or greening with or without slight clearing								No growth					
Identification	*Str. bovis*								*Str. thermophilus*					

Characters common to all strains

Growth occurs at 45° C.; resist heating for 30 min. at 63° C.; sucrose and lactose are fermented; there is no detectable action on glycerol, arginine, hippurate, gelatin or casein; litmus milk is acidified and in most cases clotted, and the reduction of litmus is weak or absent; neither acetoin nor CO_2 are formed from glucose or citrate; growth does not occur in 6·5% NaCl broth or in Foster, Garey & Frazier's(9) lactate medium.

been separated into the types A to N. It should be mentioned that the number of cultures included in each type has no statistical significance. Any organism that appeared to be atypical was purposely selected for this part of the work. Furthermore, the series is likely to contain several cases of a strain being duplicated owing to an organism having produced more than one colony on the plate used for the original isolation.

The table shows that the strains fall into two main groups comprising respectively the types A to H and I to N. The two groups are distinguished absolutely by several characters and to varying degrees by others. Type A corresponds in every respect to a typical *Str. bovis* and type I to a typical *Str. thermophilus*.

In assessing the relationships to *Str. bovis* of types B to H, no importance can be attributed to the variations in diastase production and fermentative activity; all have been reported previously (Sherman (1)) for *Str. bovis* and its varieties. Inability to ferment salicin is apparently an exceptional feature in this species. Sherman (1) records it as an occasional variation of the non-diastatic variety only. Of 513 cultures tested for salicin fermentation in this work only the three of type D (which are diastase producers) failed to form acid.

The remaining variations shown by types C to H are departures from the present conception of *Str. bovis*. The number of cultures failing to attack aesculin is noteworthy for Sherman (1) has apparently not encountered this deviation in *Str. bovis*. It may be a peculiarity of strains derived from milk not shown by those found in the alimentary tract. The occurrence of such organisms in milk powder has been recorded by Nichols (3).

Types F, G and H show in comparison with the typical *Str. bovis* a considerably wider range of temperature permitting growth and a greater indifference to the inhibitory agents salt and methylene blue. Particular attention has been given to the lower temperatures because of their importance in controlling the organisms in market milk. Every culture of A to H is able to multiply at 22° C. The growth of E to H at lower temperatures is described in Table 2, where 'slow' signifies approximately 7 days and 'very slow' approximately 14 days as the periods required for the production of a definite colour change in litmus milk. The findings differ from those of Orla-Jensen (8) who originally described *Str. bovis* as having a maximum temperature at about 45° C. and a minimum temperature above 22° C., although he mentioned two variants with a wider range of growth temperature. His observation that the ability to grow at 45° C. is an unstable property has not been repeated with any strain discussed here. Many have been retested, in some cases up to 4 years after isolation, and they have consistently shown growth at 45° C. Foster, Garey & Frazier (9) record the minimum temperature for *Str. bovis* as 10–15° C., but the identity of their strains is uncertain as some are stated to form ammonia from peptone.

The growth of F, G and H in 4% salt broth and of G and H in 0·1% methylene blue milk is markedly restrained but is quite definite. In the last-mentioned medium G and H show in 6–7 days at 37° C. yellowish white areas, and in 12–18 days the dye is reduced at the base and the milk is clotted. These results have been repeatedly confirmed, in some cases by plating the curdled milk cultures, isolating colonies, and identifying the resulting cultures with the organisms originally inoculated.

Types F, G and H deviate from the typical *Str. bovis* in the direction of the enterococci. Of interest in this connexion are the reports by Sherman (10) and Shattock & Mattick (4) that certain strains of *Str. bovis* show a serological relationship to Lancefield's group D. It should be emphasized, however, that F, G and H, the extreme variants in our series, are easily distinguishable from enterococci by diastase production, inactivity towards arginine, aesculin and citrate, inability to form acetoin from glucose or to proliferate in the presence of 6·5% salt, and by other features.

The findings just described do not indicate any clear lines of division among the types A to H. They suggest rather that the characterization of *Str. bovis* should be broadened so as to embrace all the variations shown by these strains.

The organisms described under I to N in Table 2 form a relatively homogeneous and distinctive group. There is no difficulty in recognizing I as the typical form of *Str. thermophilus*, and since J to N differ only slightly they have been allotted to the same species.

In addition to the characters mentioned in the table, all the strains possess the exacting nutritive requirements and the ability to produce on solid media only the very meagre growth characteristic of *Str. thermophilus*. No growth was detected on blood agar, apparently because this medium does not satisfy the need of a fermentable sugar. The minimum temperature for growth falls between 16 and 22° C. In experiments on the multiplication of bacteria in pasteurized milk, which will be described in another paper, 18° C. was the lowest temperature at which the proliferation of *Str. thermophilus* was detected.

Wright (11) and others have described a peculiar feature of *Str. thermophilus*; sucrose and lactose are utilized more readily than the hexoses. The relative availability of glucose and the two disaccharides to the organisms here described was tested in a broth containing 0·5% meat extract, 0·5% peptone and 1% tryptone to which was added 0·5% sugar from an autoclaved aqueous solution. The media were inoculated with a straight wire from 18 hr. lactose broth cultures. After overnight (16–18 hr.) incubation at 37° C. types I to N showed good growth and acid formation in the sucrose and lactose broths and little or no sign of activity in the glucose medium. In contrast, *Str. bovis* (A to H) multiplied and formed acid actively in all three broths during the same period. Changes in the fermentative powers of pure cultures of lactic acid bacteria have frequently been observed, and Orla-Jensen (8) and Wright (11) report their occurrences in *Str. thermophilus*. The work of these authors suggests that the method of differentiation just described could not be expected to give reliable results if it were applied to cultures that had been propagated for some time on a medium in which glucose is the source of energy.

All the deviations from Sherman's (1) characterization of *Str. thermophilus* shown for types J to N in the table have been noted previously by others. Orla-Jensen (8), Wright (11) and Foster, Garey & Frazier (9) mention maximum growth temperatures under 50° C. The fact that all the strains studied by Sherman & Stark (12) proliferated at 50° C. may perhaps be due to a selection of such types by the method of isolation employed. The growth of certain strains of *Str. thermophilus* in media containing 2% NaCl is described by Orla-Jensen (8) and Nichols (3). It will be noted that 3% NaCl completely inhibits every strain studied here. Sherman's observation that this species, in comparison with other streptococci, is especially sensitive to salt has thus been confirmed.

The fermentation of maltose by certain strains is reported by Orla-Jensen (8), Hucker (13), Wright (11), Burri (14) and Burri & Elser (15). In most cases the acid formation is described as weak, and the available evidence indicates that inactivity towards this sugar is a valuable diagnostic feature of *Str. thermophilus*. The strains placed under J, K and L in the table never showed acid production in maltose broth before 36 hr. at 37° C. and the pH never dropped below 5·6, a feature which is easily recognized if the medium contains bromcresol purple. This is in contrast to *Str. bovis* which produces a greater acidity within 24 hr.

With due regard to the variability of fermentative activity among the lactic acid bacteria, and especially to the variability of *Str. thermophilus* described by Burri (14) and Burri & Elser (15), the conclusion may be drawn that this species can be distinguished with reasonable accuracy from *Str. bovis* by testing for the fermentation of both maltose and salicin (the test substances having been sterilized in aqueous solution, preferably by filtration). The results require to be interpreted in the knowledge that uncommon strains of *Str. bovis* fail to ferment salicin, and that occasional strains of *Str. thermophilus* produce a late and weak fermentation of maltose and perhaps of salicin also. This short method of

differentiation was applied to the large number of cultures of these organisms that were isolated from milk during the course of this work.

Str. thermophilus has the reputation of being exceedingly delicate. This characteristic seems, however, to be related only to certain conditions which happen to be less harmful to other streptococci. To one inimical factor, heat, it is possibly the most resistant streptococcus known; and its common occurrence on dairy equipment indicates a considerable measure of hardiness. The difficulty of maintaining stock cultures, which is often mentioned, appears to be largely attributable to unsuitable methods. Milk is probably unsurpassed as a medium for the organism, and no difficulty has been encountered in this work in propagating stock cultures in litmus milk with transfers every 2 months. The only precautions which need to be observed are to remove the cultures from the incubator as soon as the first change in reaction is detectable, and to hold them thereafter at a temperature below 15° C. and under conditions that do not permit rapid evaporation. Cultures in milk + chalk in screw-capped bottles may remain alive for over a year if treated in this way.* If cultures in ordinary litmus milk are kept in the incubator until coagulation has occurred they usually fail to transfer within 3–4 weeks. The implication is that acid is the main factor bringing about death in milk.

Hucker (13) has stated that *Str. thermophilus* is easily killed by exposure to low temperatures for short periods. Since this information, if it were well founded, might have practical applications, some contrary evidence which we obtained should be recorded. In the routine test for growth at 8–12° C., which was applied to all streptococci isolated in this work, it was the practice to confirm negative results (after 2–3 weeks) by placing at 37° C. any tubes of litmus milk in which no change appeared. With few exceptions the dormant cultures of *Str. thermophilus* showed prompt growth.

THE *STR. LACTIS–STR. CREMORIS* GROUP

The identification of organisms of this group has presented no difficulty. Cultures placed here by the characters shown in Table 1 were further tested for the ability to proliferate at 40° C. (in a thermostatically controlled water-bath). In every instance those that formed ammonia from arginine produced obvious changes in litmus milk at 40° C. and were identified as *Str. lactis*; those that did not form ammonia failed to show growth at 40° C. and were classified as *Str. cremoris*. Representative strains were examined more fully, and they did not deviate in any way from Sherman's (1) and Yawger & Sherman's (16) characterizations of the two species. It may be noted that no strain showed growth in 6·5% salt broth or attacked gelatin, raffinose, glycerol, sorbitol, inulin, starch or citrate. The strains of *Str. lactis* fermented maltose and were variable in their action on sucrose and mannitol. The strains of *Str. cremoris* failed to ferment either maltose or mannitol and showed variation in their action on sucrose and aesculin and in their ability to grow in 0·1% methylene-blue milk.

The heat resistance of the organisms of this group, a matter of considerable importance in relation to the commercial treatment of milk, appears to deserve further clarification. It seems not unlikely that much of the apparently contradictory evidence which has been published on the question can be attributed to a variation in the methods by which the property was measured. When the strains described here were tested for resistance to heat

* These methods are also useful in maintaining stock cultures of all the other types of streptococcus discussed in this paper. Milk + chalk is superior to cooked meat as a medium for preserving *Str. bovis* and *Str. thermophilus*.

by the method already detailed it was noted that in occasional trials suspensions which had been exposed to 60° C. for 30 min. would yield on plating a small number of colonies (not more than six). Such a result could be obtained only erratically with any strain, and when the survivors were further examined they showed, in comparison with the parent culture, no increase in heat resistance. When a temperature of 63° C. was employed survival could in no instance be detected by plating. On the other hand, when the milk suspensions which had been heated at 60° C. were incubated, strains of both *Str. lactis* and *Str. cremoris* not uncommonly, but again erratically, showed growth. Less frequently growth occurred in the milk after heating at 63° C. These findings indicate that an exceedingly small proportion of the cells remains viable even at the lower of the two temperatures. Evidence corroborating the results obtained with pure cultures has been secured in the laboratory pasteurization of samples of market milk. At this point it is perhaps worth mentioning that resistance to heat, measured by the method used in this work, provides a very clear-cut distinction between *Str. lactis* and the enterococci.

Str. faecalis and its varieties

Cultures that have been identified as *Str. faecalis* and related forms were as a rule easily recognized by the characters shown in Table 1. The sole irregularity encountered in the primary grouping was the apparent inability to proliferate at 45° C. of organisms that were isolated on two occasions. In litmus milk an unexpectedly large proportion of the cultures failed to show the vigorous reduction characteristic of most enterococci, and one strain was incapable of producing any naked-eye change in that medium. Every culture of this group was tested in gelatin and in sucrose, glycerol, mannitol and sorbitol broths. An adequately representative series was found to be uniform in the properties noted at the foot of Table 3. Features in which the strains differ are also shown in the table. The

Table 3. *Characters of* Str. faecalis *and related organisms*

	A	B	C	D	E
Growth at 45° C.	+	+	−*	+	−*
Growth in presence of 6·5% NaCl	+	+	+	+	Slight
Gelatin liquefaction	−	+	−	−	−
Hippurate hydrolysis	±	+	+	+	+
Acid production from:					
Sucrose	+	+	+	−	−
Glycerol	+	+	+	∓	−
Mannitol	+	+	+	−	−
Sorbitol	+	+	+	−	−
Fermentation of citrate	+	+	+	+	−
Litmus milk†	*RC*	*RCW*	−	*C*	*C*

* Growth at 45° C. has occurred in glucose yeast extract milk in a proportion of trials.

† *RC* = vigorous reduction and acid clot. *W* = whey separation. *C* = acid clot and weak or no reduction of litmus. − = no change.

Characters common to all strains

Growth occurs at 8–12° C.; resist heating for 30 min. at 63° C.; growth is not prevented by 0·1% methylene blue in milk or 40% bile in broth; ammonia is formed from arginine; acetoin is formed from glucose; lactose and aesculin are attacked; raffinose and inulin are not fermented; diastase is not produced; there is no β-haemolysis.

variations do not appear to be sufficiently important to exclude any of the organisms from the enterococcus group. The atypical forms characterized under C, D and E in the table possess the heat resistance associated with the group; they all survived heating at 63° C., and representative cultures which were tested at 65° C. withstood that temperature for 30 min. We are greatly indebted to Dr P. M. F. Shattock for making a serological

examination of eight cultures of type D and two of type E. She reported that they all belong to Lancefield's group D and that extracts prepared from type E do not react with group N (*Str. lactis*) serum. These findings provide additional evidence that the organisms may be accepted as enterococci.

Type A conforms exactly to *Str. faecalis* and B is typical of *Str. liquefaciens*. Type C has the unusual feature of producing no obvious change in litmus milk. The organism, nevertheless, multiplies quite extensively in that medium; microscopical counts greater than 10^8/ml. are obtainable in 24 hr. at 30° C. Lactose is fermented in broth, though less actively than glucose (from which a final pH of 4·4 is produced) and the addition of glucose to milk results only in slight acidification. The inactivity in milk cannot therefore be attributed simply to an inability to attack the sugar. Possibly another peculiarity of this strain, the difficulty with which growth occurs at 45° C., is due to the same metabolic deficiency. Since the organism has not been found to differ in any other character from a typical *Str. faecalis* it may be regarded as a variant of that species. Type C was isolated only once. It was detected in milk that had been pasteurized in the laboratory and then held at 17° C. for 8 days. Colonies of the organism (10 were transferred and identified) were obtained on MacConkey's agar.

Type D shows the fermentation reactions and the behaviour in litmus milk characteristic of *Str. durans*. Only 7 of the 328 cultures examined ferment glycerol and they produce a slow and weak fermentation of that substance. Type D differs from *Str. durans* in being non-haemolytic. On horse or ox-blood agar the deep colonies produce nothing more than a slight clearing with a hazy margin of greening. The organisms are thus intermediate in character between *Str. durans* and *Str. faecalis*. There is ample evidence in the literature supporting the view that the last-mentioned species should embrace strains which do not ferment one or more of the four differential substances, sucrose, glycerol, mannitol and sorbitol, or do not give the typical reaction in litmus milk. Thus type D might well be regarded as a variant of *Str. faecalis* which is deficient in all five properties. At the same time, the 328 cultures examined show uniformity of character to a somewhat exceptional degree, and an ecological observation further suggests that they represent a distinctive type or variety: they were rarely detected in milk either before or immediately after pasteurization, but when the pasteurized milk was held at 17° C. they not uncommonly became the dominant organisms. Under these conditions they appear to proliferate more rapidly than the typical *Str. faecalis*. Strains of similar character but somewhat more variable in their fermentation reactions were found by Nichols (3) to be prevalent in dried milk powder.

Type E has been encountered only once: it was the dominant organism (60 of its colonies were identified) in a sample of laboratory pasteurized milk after storage at 17° C. for 10 days. Table 3 indicates that it is an exceptional form in the enterococcus group. Although it multiplies freely in litmus milk if the temperature is not above 37° C. it has failed to grow in that medium at 45° C. except from large inocula, and then only erratically. In glucose yeastrel milk, the most favourable medium yet found, the initiation of growth at 45° C. is dependent on the source from which the transfer is made—e.g. milk cultures yield growth while broth cultures do not. An additional peculiarity is its poor lactate tolerance; unlike types A to D it fails to grow in Foster, Garey & Frazier's (9) lactate medium.*

* When used in the differentiation of streptococci this medium appears to test lactate tolerance rather than lactate utilization. Its growth-promoting quality for enterococci is not reduced by omission of the lactate.

In a previous paper (Gibson & Abd-El-Malek (6)) a technique was described whereby the fermentation of citrate by streptococci could be detected visually through the liberation of gas. Examination of representatives of several homofermentative species showed that the ability to ferment citrate with gas formation was confined to members of the enterococcus group. Two strains which appeared to be atypical enterococci failed to show evidence of citrate utilization. One, the strain J.M.A. 3 described by Shattock & Mattick (4), differs from a typical *Str. faecalis* in showing a very low percentage survival when heated at 60° C. for 30 min., in being markedly inhibited by 6·5% NaCl, in failing to ferment glycerol and sorbitol, and in its slow acid formation and weak reducing activity in litmus milk. The second strain is described under E in Table 3.

The organisms tested for gas formation from citrate have also been examined for the production of acetoin from the same substance. The medium finally employed for the latter purpose was 1% peptone, 0·25% yeastrel and 1% of a mixture of 9 parts tribasic potassium citrate and 1 part citric acid. Cultures were tested for acetoin with Barritt's (17) reagents. Preliminary trials showed that 1% citrate mixture yielded clearer results than 0·5%. They also showed that the reaction of the medium has an important influence. The optimum appeared to occur between pH 5 and 6; media with an initial pH of 7 gave weaker and erratic results. These observations have now been clarified by the detailed study by Gunsalus & Campbell (18) of the effect of reaction on the citrate fermentation of a strain of *Str. faecalis*.

This method of detecting the utilization of citrate gave, in the case of the homofermentative streptococci, results identical with those afforded by the test for gas production; and it has the advantage of being the quicker method. Heterofermentative streptococci, as would be expected, failed to produce acetoin in the sugar-free citrate broth. They showed no indication of growth in the medium.

These findings added the inability to ferment citrate to the other aberrant properties of strains E and J.M.A. 3 and suggested that the organisms should be set apart from other members of the enterococcus group. The significance of citrate fermentation in this group and the apparently unique character of the two strains are now more uncertain, for Campbell & Gunsalus (19) have since reported a strain of *Str. faecalis* which does not utilize citrate. Tentatively, it may be concluded that the fermentation of citrate is an important property of the enterococci, perhaps no less regular in its occurrence than several other characters that have proved to be useful in delimiting the group.

The streptococci tested for activity towards citrate were also examined for the production of acetoin in glucose broth. Positive results were obtained with all the enterococci with the exception of a typical strain of *Str. durans*. The formation of acetoin from glucose is thus, like the fermentation of citrate, a normal but not a universal property in the enterococcus group. The only other species in which it was detected are *Str. lactis* and *Str. cremoris*, of which some but not all strains gave positive results.

The taxonomy of the enterococcus group is a debatable question. The evidence presented in this paper supports the opinion that on account of the intergradation which exists, the subdivisions of the group so far defined do not constitute convenient species. For this reason we prefer to regard the several types described in preceding paragraphs as varieties or variants of one species, *Str. faecalis*; and they will be so treated in subsequent discussions.

HETEROFERMENTATIVE STREPTOCOCCI

In comparison with the homofermentative species the heterofermentative streptococci were much less easily identified. An initial problem was to find a simple method by means of which they could be recognized. This was solved when the cultural test for CO_2 formation had been devised. The further problem, the specific identification of the organisms, presented complications, for in this group the delineation of species has not progressed far beyond the use of fermentation reactions; and the available evidence strongly suggests that such criteria have here the same limited significance that they have among the homofermentative groups. In 1919 Orla-Jensen (8) realized that in defining species he was working on treacherous ground, and it is questionable if the ground has in the meantime become much firmer.

In an attempt to identify the species isolated from milk a sample of 40 cultures was examined in some detail. Table 4 shows the characters of the organisms. Clearly, there

Table 4. *Characters of heterofermentative streptococci*

	A	B	C	D	E	F	G
No. of cultures	4	3	14	3	2	3	11
Growth at 37° C.	−	−	+	+	+	+	+
Survival at:							
60° C. 30 min.	−	−	+	+	+	+	+
63° C. 30 min.	−	−	±	±	±	±	±
Acid production from:							
Lactose	+	−	+	+	+	+	+
Maltose	+	Slight	+	+	+	+	+
Sucrose	−	−	+	+	+	+	+
Raffinose	−	−	+	+	−	−	+
Salicin	−	−	−	+	−	+	−
Arabinose	−	−	−	−	−	−	+
Litmus milk	Trace of acid	No change	Acid or acid clot; little or no reduction				
Identification	*Str. citrovorus*		*Str. kefir*				

Characters common to all strains

Growth occurs (slowly) at 8–12° C. but not at 45° C.; acid is formed from glucose; xylose, mannitol and sorbitol are not fermented; sucrose gelatin cultures show no dextran synthesis; CO_2 is formed actively from glucose and citrate; there is no hydrolysis of arginine, hippurate or aesculin and no β-haemolysis.

are two main groups, the one consisting of types A and B, the other of C to G. In each of the two groups the strains appear to be essentially alike; no distinctions other than in fermentation reactions were detected in either group. For this reason, a separation of the strains into more than two species on account of a difference in their action on arabinose, or any of the other test substances mentioned in the table, appears to serve no useful purpose. It will be noted that none of the strains gave any naked-eye evidence of synthesizing dextran from sucrose. Attempts to demonstrate this action were made with gelatin and agar each containing 5% sucrose. The addition to these media of yeast autolysate or tomato juice and, in the case of certain strains, repeated subculture in sucrose-containing media were also tried, but in no instance did the growth show a mucoid character.

Types A and B are identifiable with *Str. citrovorus* Hammer (20). This species has attracted attention on account of its occurrence in dairy starters and its association with the aroma of diacetyl in butter. Organisms possessing the general character of *Str. citrovorus* have been studied especially by Boekhout & Ott de Vries (21), Hammer (20),

Ayers & Mudge (22), Orla-Jensen, Orla-Jensen & Spur (23), Knudsen & Sörensen (24) and Hucker & Pederson (25), with the result that it is probably the most clearly defined species of heterofermentative streptococcus. It is distinguished by a low maximum temperature, a restricted range of fermentable substances, the pentoses and one or more of the disaccharides (sucrose, maltose or lactose) being unavailable, and an inability to produce in litmus milk more than a faint change in colour. The strains obtained from milk in this work are entirely typical. The lactose-fermenting type (A) showed in litmus milk nothing more than the slightest discernible acidity after 14 days at 30° C. The addition to the medium of yeast autolysate or tomato juice promoted acid formation but resulted in little or no clotting. None of the pure cultures survived heating for 30 min. at 60° C. although on one occasion type B was found in a sample of laboratory pasteurized milk after storage.

The naming of types C to G is less straightforward. They conform exactly to the characterization of *Str. kefir* Migula as emended by Evans (26) and to the additional features of that species described by Ayers, Johnson & Mudge (27). *Str. paracitrovorus*, which was described in somewhat greater detail but at a later date than that of Evans's publication, is generally accepted as a synonym. Hucker & Pederson (25) regard both species as identical with Beijerinck's (28) *Lactococcus dextranicus*. The strains described here differ, however, from Beijerinck's organism in their failure to split aesculin or to synthesize dextran from sucrose. While the significance of these characters is at present uncertain they may be of importance, and until the matter is clarified it is perhaps premature to discard *Str. kefir* as the name for the prevalent type of heterofermentative streptococcus in milk.

The resistance to heat of *Str. kefir* and related organisms has apparently never been studied systematically. Orla-Jensen (8) and Hammer & Baker (29) report a relatively high level of resistance in the case of certain strains. The cultures of *Str. kefir* isolated by us invariably survived heating for 30 min. at 60° C. but with a considerable reduction in the number of colonies obtainable on plating. After heating at 63° C. the suspensions produced either no colonies or only a small number.

The distribution of the species in milk

In Table 5 is shown the number of samples of milk in which the individual species of *Streptococcus* were detected. The milk was partly bottled herd supplies and partly bulked milk. Data concerning the udder samples has been excluded. The last column refers to milks pasteurized in the laboratory and then held at various temperatures between 10 and 22° C. (at about 17° C. in the majority of cases) until a taint and other signs of bacterial action had appeared.

There are several limitations to the significance of the figures in the table: (1) the relative abundance of the species in the samples is not shown; (2) the organisms recorded are only those that occurred in sufficient numbers to be represented on the plates; (3) the samples were not investigated in equal detail; the plates of raw milk were incubated only at 30° C. while both 30 and 37° C. were used for many of the samples of pasteurized milk (fresh and tainted). Incubation at 37° C. was introduced in order to increase the chances of demonstrating thermoduric streptococci in the presence of a large population of other bacteria. The table nevertheless affords evidence on the distribution of the species. Thus, in the raw milks *Str. kefir*, *Str. lactis* and the mastitis organisms were detected most

frequently. Pasteurization simplified the streptococcal microflora so that in the majority of instances *Str. thermophilus* and *Str. bovis* were the only species identified. The results obtained with both the raw and the pasteurized milk are very clearly related to the conditions under which the milk was produced and transported. This matter will be taken up in a later paper. The species that have been observed to proliferate in pasteurized milk are shown in the last column of the table. Here again the differential effect of the heat treatment is obvious but, in addition, the outcome depended in part on whether the temperature of storage was above or below the minima for *Str. bovis* and *Str. thermophilus*.

Table 5. *The number of samples of milk in which each species of* Streptococcus *was detected*

	Raw milk	Pasteurized milk when fresh	Pasteurized milk when tainted
No. of samples examined	23	54	28
No. of samples yielding streptococci	22	33	21
Mastitis group	16	—	—
Str. bovis	2	25	10
Str. thermophilus	—	28	5
Str. lactis	17	—	1
Str. cremoris	10	—	—
Str. faecalis	7	6	9
Str. kefir	19	5	13
Str. citrovorus	3	—	1

A notable feature of these results is the prevalence of *Str. kefir*. This organism was isolated from all classes of raw milk with greater frequency than *Str. lactis* and, although it suffers a severe mortality during pasteurization, it not uncommonly becomes the dominant streptococcal species in pasteurized milk kept at 10–22° C. Ayers, Johnson & Mudge, in a paper (27) which seems to have been largely overlooked, have previously presented evidence of the prevalence of *Str. kefir* in raw milk. The fact that the organism produces a relatively slow colour change in litmus milk may perhaps have led to the supposition that its growth and activity in market milk could have little significance.

SUMMARY

The streptococci occurring in raw and pasteurized milks of varying purity have been investigated. The organisms were placed in five main groups by testing for the hydrolysis of arginine, growth at 45 and at 8–12° C., survival in milk at 63° C. for 30 min. (measured by a semi-quantitative plating method), action on litmus milk, and the ability to form CO_2 from glucose (tested by a cultural method). The groups thus distinguished are:

(1) Organisms of bovine mastitis.

(2) *Str. bovis* and *Str. thermophilus*. A clear-cut separation of these species was not obtained by tests for growth at 50° C., the hydrolysis of aesculin, or the fermentation of maltose or salicin; but, with suitable precautions, action on both maltose and salicin provides a differentiation. Certain strains of *Str. bovis* differ from the typical form of that species in their ability to proliferate over a wider range of temperature and in higher concentrations of salt and methylene blue and in their failure to split aesculin.

(3) *Str. lactis* and *Str. cremoris*, which were differentiated by tests for the hydrolysis of arginine and ability to grow at 40° C.

(4) *Str. faecalis* and its varieties. Many of the strains are non-haemolytic but otherwise indistinguishable from *Str. durans*. The formation of acetoin from citrate and from glucose are common but not universal properties in the enterococcus group.

(5) Heterofermentative streptococci. The majority of the strains were identified as *Str. kefir*. *Str. citrovorus*, which was isolated only occasionally, was distinguished by its low maximum temperature (under 37° C.), weak resistance to heat and by fermentation reactions.

Raw milk was found to contain *Str. kefir*, *Str. lactis* and the mastitis organisms more frequently than the other species. *Str. thermophilus* and *Str. bovis* were the dominant forms in freshly pasteurized milk. Pasteurized milk held at 10–22° C. until it became tainted yielded *Str. kefir* and *Str. faecalis* and, at the higher temperatures, *Str. bovis* and *Str. thermophilus*.

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363. STUDIES IN THE BACTERIOLOGY OF MILK

II. THE STAPHYLOCOCCI AND MICROCOCCI OF MILK

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Difficulties were immediately encountered when attempts were made to identify staphylococci and micrococci isolated from milk. In this group of bacteria there is no dearth of easily recognizable characters in which strains differ; the taxonomic problem arises from uncertainty concerning the significance of the various distinctions. Except among the pathogenic staphylococci previous studies have not resulted in any noteworthy agreement on this question, and the literature provides little guidance beyond the frequently repeated findings that in pure culture certain strains do not exhibit stability in such properties as chromogenesis, liquefaction of gelatin, acid formation from several test substances, coagulase activity and toxin production.

Hucker (1, 2, 3), in an investigation of a comprehensive series of cultures from dairy products and many other sources, reached the conclusion that the species could be differentiated by chromogenesis, gelatin liquefaction, nitrate reduction and ability to grow with an ammonium salt as the only source of nitrogen. As his methods have been utilized by later workers, and as they afford a simple and clear-cut separation of species, they were employed at the outset of the present work. It soon became obvious, however, that Hucker's differential criteria are distinctly artificial; they separate strains that appear to be essentially similar, and they group together types which, for some purposes at least, it is necessary to distinguish. An example of the confusion to which they may give rise is to be found in the literature. By the use of these methods of identification the micrococci occurring in the cow's udder (Breed (4)) have been given the same names as the thermoduric types found in pasteurized milk (Robertson (5), Hucker (6), Prickett (7), Hileman, Leber & Speck (8)). This appears to be the origin of an erroneous assumption that the udder is an important source of the heat-resistant micrococci of milk (Hileman (9), Hileman, Leber & Speck (8), Fabian (10), Dotterrer (11), Fabian (12)).

The work to be described was done with the purpose of obtaining information about the main types of staphylococci and micrococci that occur in milk. From among cultures which had been subjected to only a few diagnostic tests a series of 598 was selected for further study. In making the selection, representatives of any variations in character and source of the organisms were included as far as possible. The selected cultures, which are the subject of this paper, were replated before being examined in greater detail. They had been isolated as follows: 248 from the cow's udder,* 76 from raw milk, 242 from pasteurized milk and 32 from pasteurized rinsings of dairy equipment. For purposes of comparison additional cultures were examined. They include 10 named cultures from the N.C.T.C., 57 cultures from the human body (skin and infections) and 134 from animals, principally sheep. The animal strains were received from workers at the Moredun Institute who had already tested most of them for pathogenicity to the mouse.

* We are indebted to Dr A. Cunningham for specimens of milk taken with aseptic precautions.

Methods

Some of the findings of this inquiry may not be reproducible if the laboratory methods on which they are based should be modified. Accordingly, such details as appear to be necessary will be given. Several tests, although they may possess little differential value, will be mentioned either because of their relation to previous work by others or because the action of the organisms detected (e.g. on casein, lactose and fat) might be of importance in dairy products.

Tests of resistance to heat. The method used was the same as that previously described (Abd-El-Malek & Gibson (13)) for the examination of streptococci except that the cells to be heated were grown in sugar-free broth. The essential features of the procedure are the heating in milk and the immediate plating of the heated suspension. A method which is at least semi-quantitative is required in order to secure consistent results.

Hydrolysis of arginine. Niven, Smiley & Sherman's (14) method as modified previously (13) was used. Cultures were incubated for 3 days at 37° C. or 5 days at 30° C. Staphylococci and micrococci may form small amounts of ammonia from the yeast extract of the medium, and doubtful reactions were controlled by parallel cultures in a medium differing only in containing no added arginine.

Coagulase production. Rabbit plasma was used in the method recommended by Chapman, Berens & Stiles (15). The majority of the strains were also examined by a rapid slide test (Berger (16)) using fibrinogen prepared from rabbit plasma. The two methods gave the same result in every instance in which they were compared. The use of whole plasma in a slide test was also tried, but was given up because of the agglutination of occasional cultures which were negative in the tube and the fibrinogen tests.

Haemolysis. Plates of 5% sheep- or ox-blood agar, inoculated on the surface, were incubated for 48 hr. at the optimum temperature of the organism and were then placed in the refrigerator overnight.

Fermentation tests. Cultures in peptone 1%, test substance 0·5%, with bromcresol purple as indicator, were incubated up to 7 days at the optimum temperature. Pentoses were sterilized in aqueous solution (10%) at 15 lb. for 15 min.

Acetoin formation. Cultures in peptone 1%, glucose 0·5% were incubated at 37° C. and tested by Barritt's (17) method after 3 days and, if negative, after 5 days.

Diastase production. Plates of nutrient agar + 0·2% soluble starch, inoculated on the surface, were incubated at 30° C. for 7 days and were then flooded with iodine.

Nitrate reduction. Cultures in peptone 1%, KNO_3 0·1% were tested for nitrite and, if negative, for nitrate.

Growth with ammonia as nitrogen source. The ability to grow with an ammonium salt as the only source of nitrogen was tested in the medium used by Hucker (1) and, for comparison, in another of a similar type.

Gelatin liquefaction. Stab cultures in nutrient gelatin were incubated at 22° C. In order to secure data which could be compared with reports by other workers, the tubes were closed with stoppers and, if liquefaction failed to occur, were incubated for several months. The usefulness of such long incubations seems, however, to be extremely doubtful.

Casein digestion. Plates of (*a*) nutrient agar + 20% skim milk, and (*b*) a casein agar (18) were inoculated on the surface. After incubation (*a*) was flooded with 10% HCl and (*b*) with 1% tannic acid.

Fat hydrolysis. Plates of nutrient agar containing 5% emulsified fat (butterfat, cotton-seed oil or olive oil) were inoculated on the surface, and after 7 days at 30° C. they were tested with $CuSO_4$ (Berry (19)).

PRIMARY GROUPING

The distinction between *Staphylococcus* and *Micrococcus* is at present quite indefinite. These names have been used in various senses and often indiscriminately, especially with reference to the apparently harmless parasites of the animal body. Some evidence bearing on the question will be presented, but the problem will not be discussed specifically in this paper. The dividing line between *Micrococcus* and *Sarcina* is equally obscure. Many of the strains to be described produce tetrads, often groups of tetrads lying in one plane, but after some fruitless attempts to demonstrate fission in three directions of space, a feature which certain sarcinae exhibit only under special conditions (20), the practice adopted was to regard as a micrococcus any organism that failed when growing on agar to show three-dimensional bales of regular form.

Table 1. *Differential characters of the staphylococci and micrococci of milk**

	I Staphylococcus group				II Intermediate group	III Dairy micrococci	
	A	*B*	*C*	*D*		*A*	*B*
Acid from glucose	+	+	+	+	−	+	+
NH_3 from arginine	+	+	+	−	−	−	−
Acetoin formation	+	+	+ or weak	− or weak	−	−	−
Coagulase production	+	−	−	−	−	−	−
Acid from mannitol	+	−	V	V	−	−	−
Survival in milk at 60° C. 30 min.†	−	−	−	−	V	+	+
Acid from glycerol	V	V	V	V	−	−	+
Pigment on agar	Yellow, orange, fawn or none				Greenish yellow or none	Greenish yellow	Pale greenish yellow or none
Principle source	Animal body				?	Dairy equipment	

V = variable.

* Uncommon exceptions are mentioned in the text.

† Strains showing slight survival, as indicated by the occasional production of a small number of colonies (< 10) when the heated suspension is plated, are regarded as being killed (−).

The observations to be described have indicated that several features which distinguish individual strains of staphylococci and micrococci are so slightly correlated with any other attribute of the organisms that they are unlikely to possess much taxonomic utility. Examples are the ability to liquefy gelatin and to reduce nitrate, characters to which considerable attention has often been given. When this situation became apparent the *Staphylococcus-Micrococcus* assemblage began to appear less like a complex mosaic. The pattern could be simplified to that of a linear series. At one extreme is the pathogenic staphylococcus and at the other a thermoduric saprophyte. Although the series appears to be essentially continuous, three main divisions may be recognized. Some of the distinctions between the three groups, and the subdivisions that have been noted in two of them, are shown in Table 1.

Group I consists of organisms which are relatively sensitive to heat and which ferment glucose and other sugars. They are, mainly at least, parasitic and some or all are often

classified as staphylococci. They will be referred to here as the staphylococcus group. The hydrolysis of arginine and the formation of acetoin by organisms of this group have previously attracted little interest. Both appear to be valuable differential criteria.

Group II comprises the obligate aerobes which do not ferment sugars. The organisms have been isolated from a variety of situations but in numbers too small to justify conclusions concerning their habitats. In character they are intermediate between groups I and III.

Group III, the thermoduric sugar fermenters, appear to be strict saprophytes. They occur with such frequency on imperfectly sterilized milk utensils that they deserve recognition as the micrococci typical of the dairy. This group divides naturally into two types which may be distinguished by their action on glycerol and in other ways.

I. The staphylococcus group

This group, although it appears to be a series of types in which there are no very sharp lines of cleavage, is divided in Table 1 into four subgroups. This has been done partly for practical reasons, such as the importance of defining the pathogenic forms, and partly to bring out the gradual transition in character from *A* to *D*. Other properties of the organisms are shown in Table 2. Subgroup *A* is regarded as comprising pathogenic

Table 2. *Characters of group I*

	A	*B*	*C*	*D*
No. of cultures	328	57	72	22
Pigment production on milk agar and potato	321+ 7−	23+ 34−	38+ 34−	7+ 15−
Haemolysis*	323+ 5−	2+ 51− (4 not tested)	10+ 62− or slight	−
Nitrate reduction	+	51+ 6−	48+ 24−	13+ 9−
Growth with NH_3-N	−	−	−	7+1?14−
Gelatin liquefaction (22° C. 4 weeks)	323+ 5−	34+ 23−	49+ 23−	14+ 8−
Casein digestion	−or weak	−or weak	40+ 32−	9+ 13−
Acid from lactose	326+ 2−	46+ 11−	62+ 10−	19+ 3−
Fat hydrolysis	6+ 321− or weak	51+ (6 not tested)	27+ 45−	−
Tributyrin hydrolysis	+	51+ (6 not tested)	+	2 weak+ 20−
Final pH	4·6–4·8	4·6–4·8	4·8–5·2	5·2 or higher
Anaerobic growth in glucose agar	+	+	+	+ or −

* In *B* and *C* no darkened zones occurred and + = clear zone only.

staphylococci. *B*, *C* and *D* appear to be in the main harmless parasites of the animal. The cow's udder provided a large proportion of the cultures in each subgroup (149 in *A*, 44 in *B*, 39 in *C* and 14 in *D*). The remaining cultures were obtained from raw market milk and a variety of animal and human sources.

Some difficulty has been experienced in the selection of a heat treatment that will invariably distinguish all strains of group I from all strains of group III. When freshly isolated cultures were tested a heating in milk at 63° C. for 30 min. provided a clear-cut distinction, but when this treatment was applied again after the organisms had been cultivated artificially for several years some of those in group III failed to survive the heating. When a temperature of 60° C. was used certain cultures of group I produced in some trials a few colonies (never as many as 10) on the plates poured from the heated

suspension. It was finally decided that 60° C. was the most suitable temperature, provided that the test is performed and interpreted as described in this paper.

An extensive literature shows that pigment production by staphylococci may be demonstrable only under special conditions, that the property is not entirely stable, and that it has only a limited significance as a diagnostic criterion. These findings have been confirmed in this study and they apply in all four subdivisions of group I. Nevertheless, the fact that in this group the pigment formation is so dependent on the conditions of growth provides, in addition to the colours produced, a means of distinguishing the other two groups. The yellow, orange and fawn tints of group I are commonly feeble or absent on meat extract (lemco) agar and are greatly intensified on potato or on agar + 20–30 % milk. In contrast, the greenish yellow pigments of groups II and III show approximately the same intensity on all three media. The differential value of the colour of the growth on meat-extract agar does not appear to have been recorded previously.

In addition to the characters described in Table 1, other features which indicate a close relationship among the organisms of group I have been noted: (1) All the cultures grow at 37° C. (2) Only 7 cultures (in subgroup *D*) have shown the ability to multiply in a synthetic medium containing no nitrogen compound other than ammonia. This confirms Hucker's (1) observation that parasitic types generally fail to grow with ammonia as the only source of nitrogen. (3) In stained preparations of agar cultures there is in the case of most strains a complete absence of the tetrads and groups of tetrads which are frequently conspicuous in cultures of groups II and III. (4) The ability to tolerate high osmotic tension is a general property of group I. With one exception every culture was capable of growth in Hartley's (21) broth to which sodium chloride had been added at the rate of 15 g. to 100 ml., provided the inoculum contained a sufficient number of viable cells. The highest concentration of salt tolerated by any strain of groups II and III was in the region of 10 %. This distinction appears to provide the basis of another differential test but we are not yet able to specify one. The medium used, the amount of inoculum and probably other factors influence the outcome in this type of test, and they require further study.

Subgroup *A* consists of organisms possessing the characters of pathogenic staphylococci. Of the 328 cultures, 143 were tested for pathogenicity to the mouse by other workers. All but two killed mice when saline suspensions were injected intravenously, although the quantity of organisms required varied from strain to strain. The data concerning pigment production and haemolysis shown in Table 2 accord with many other reports on the occurrence of these properties in pathogenic staphylococci.

Six cultures of *A* showed only a weak clotting action on rabbit's plasma. They were further responsible for most of the variations from the basic type of this subgroup. All six were lipolytic, two were non-haemolytic and two produced only a weak clearing on sheep's blood agar, two formed no pigment, and one failed to ferment lactose. The results of mouse inoculation are available for only one of the six strains; it showed no evidence of pathogenicity. A circumstance which may have a bearing on these findings is that the organisms had been more than 2 years on artificial media before the pathogenicity and the coagulase tests were made.

Subgroups *A* and *B* appear to be closely related yet they are separated almost perfectly by the coagulase test and the fermentation of mannitol. If the six weak coagulase reactions just mentioned are accepted as positive there was only a single exceptional strain: one of *A* failed to ferment mannitol. Several investigators have not obtained such a close

association of coagulase with mannitol fermentation; and in view of the numerous records of variability in these properties, and especially the finding (Blair (22), Chapman *et al.* (23)) that each may be lost independently by pure cultures, exceptions are to be expected.

The evidence presented in Table 1 suggests that a test for acetoin may be useful, especially in defining those mannitol fermenters that do not produce coagulase and are presumably non-pathogenic. Certain strains in subgroups *C* and *D* give a weak reaction in Barritt's test when the cultures are incubated at 30° C. At 37° C. they usually show no trace of reaction at any time up to 10 days, and if this temperature is used there appears to be little possibility of confusion between *A* and *B* on the one hand and *C* and *D* on the other. O'Meara's (24) medium, though not used extensively, yielded results similar to glucose peptone broth; the only discrepancies occurred with strains giving a weak reaction in one medium and none in the other. Cowan (25) reports that of 103 coagulase producers 7 gave a negative result in Barritt's test. Whether this difference in the findings is attributable to the strains examined or to some other factor, such as the length of the incubation period, is obscure.

The formation of ammonia from arginine, which was first introduced (14) for the differentiation of streptococci, promises to have applications among the staphylococci and micrococci also. As may be seen in Table 1, it distinguishes three of the four types of group I from the other two groups and, in addition, it defines in group I the type (*D*) which appears to be furthest removed from the pathogenic forms.

In an earlier paragraph it was suggested that group I constitutes a series changing in character rather gradually from *A* to *D*. This interpretation of the relationships receives support not only from the properties described in Table 1 but also from other features of the organisms.

When stained by Gram's method (using alcohol as the decolorizing agent) the cells of *A* are comparatively small (0·7–0·8μ) and are relatively easily decolorized. In the other subgroups the resistance to decolorization by alcohol and the size of the stained cell (or, more accurately, the stained part of the cell) are both greater.

The maximum temperature for growth appears to decrease on passing from *A* to *D*. The complete series of cultures has not been examined for this property, but all those of *A* and *B* that were tested are capable of proliferating at 45° C., while in *D* the maximum temperature for certain strains is little above 37° C.

The pH produced in a glucose broth (1% peptone, 1% meat extract, 0·5% NaCl, 2% glucose) was measured after 7 days at 37° C. or 10 days at 30° C. The results secured are shown in Table 2. They bring out another gradation from *A* to *D*. The fermenting power is linked with the ability to utilize glucose as a source of energy under anaerobic conditions. In glucose agar *A*, *B* and *C* behave as facultative anaerobes; some strains of *D* grow anaerobically while others (the weakest fermenters) do not.

Indications are to be found in the literature that the range of fermentable test substances decreases in passing from *A* to *D*, but this question has not been adequately explored in the present work.

Table 2 shows the distribution among the four subgroups of several characters which have been utilized by previous investigators for the recognition of species of these cocci. It also shows the action of the organisms on the main constituents of milk. In *C* and *D* an active liquefaction of gelatin is in most cases associated with caseolytic action, but in *A* and *B*, which contain the most rapid liquefiers of gelatin in group I, the digestion of

casein is at the most very slow and feeble. In *A* the action on fats is likewise absent or slight in the great majority of strains. The literature contains several reports which in the light of our experience give a somewhat misleading impression of the caseolytic and lipolytic powers of *Staphylococcus aureus*.

II. THE INTERMEDIATE GROUP

This group has been separated from the others principally on the basis of inabillty to ferment any sugar. As a consequence of this character the organisms are strict aerobes. Some additional properties of 33 cultures which were examined in detail are shown in Table 3.

Table 3. *Characters of group II*

	A	*B*	*C*
No. of cultures	12	7	14
Pigment production	−	+	+
Survival at 60° C. 30 min.	−	−	+
Gelatin liquefaction	7+ 5−	+	+
Casein digestion	7+ 5−	+	+
Fat hydrolysis } * Tributyrin hydrolysis }	−	5+ 2−	6+ 8−
Nitrate reduction	1+ 11−	−	−
Growth with NH_3-N	+	2+? 5−	+

* Every culture was either + in both or − in both.

The stability and significance of the fermentation of glucose in the staphylococci and micrococci remain to be explored. Several investigators have reported the separation from pure cultures of variants incapable of fermenting some of the sugars and other test substances attacked by the parent strain. Nevertheless, we are unaware of any instance, excepting the case of *Micrococcus tetragenus* (Reimann (26, 27)), of the ability to ferment glucose having been lost under experimental conditions. Judging the situation purely by a consideration of the properties of the cultures studied, it appears probable that group II connects the other two groups by a very complete series of intermediate forms. The cultures described under *A* in Table 3 differ only slightly from the aerobic weak fermenters of group I*D*. At the other end of the series constituting group II is the type *C* which possesses several of the characteristics of III*A*. Among these are: (1) a strong tendency for the cells to occur in tetrads and flat groups of tetrads, (2) a greenish yellow growth on solid media, (3) a considerable resistance to heat, and (4) the secretion of diastase. The heat resistance of these organisms is not of a high order; in no strain does more than a small proportion of a cell suspension survive heating in milk for 30 min. at 63° C. Even at 60° C. the survival rate may be low. Diastatic activity is also in most cases weak. One culture of *C* forms tyrosinase and imparts a dark colour to agar containing a suitable peptone.

The 14 thermoduric cultures (type *C*) were all obtained from pasteurized milk. Cultures of *A* and *B* were isolated from aseptically drawn milk, bulked raw milk and the hands. Other cultures of group II were detected in pasteurized rinsings of dairy utensils, but they were not characterized in detail and have not been included in the table.

The lemon-yellow organism known as *Staphylococcus citreus*, which may be isolated from the human body, is commonly found to be incapable of fermenting any sugar. It would thus fall into group II. *Sarcina lutea* does not differ greatly from the chromogenic strains of this group. Indeed, it is difficult to be certain that the organisms of type *C* are

quite incapable under all conditions of growth of forming the regular bales which distinguish the sarcinae. The thermoduric cocci identified as *S. lutea* by Brannon & Prucha (28) and Hammer & Trout (29) are quite possibly identical with type *C*. Robertson (5), who reported that *S. lutea* is one of the commonest thermoduric bacteria in milk, gives a description of the organism which does not conform to any of the groups discussed in this paper. The cultural characters are like those of group III *A* except that acid is not formed from sugars. There is, however, a possible discrepancy in the description: litmus milk is reported to be acidified, and this suggests the fermentation of lactose. We have examined a few cultures of the species we regard as *S. lutea* which were isolated from the air. Like group II they did not ferment glucose, but they showed neither heat resistance (at 60° C.) nor diastatic activity.

III. The dairy micrococci

The cultures of this group were in every instance obtained either from milk that had passed through the usual routine on the farm or from dairy equipment. Evidently, the equipment is the main source from which milk becomes contaminated with the organisms. The group is a characteristic component of the microflora in milk that has been contaminated from utensils and then efficiently pasteurized. A large number of cultures were isolated from these sources during the course of the work, and 284 which were selected for a detailed examination are described here.

The group is relatively homogeneous, and Table 1 shows characters which distinguish it from groups I and II. Headmarks of the organisms are the tendency for the cells to occur in tetrads, the character of the growths on meat-extract agar and the action on glucose. That combination of features is often sufficient to permit of cultures being correctly placed in the group without resort to tests of other properties. Diastatic activity was shown by every culture examined, but as a diagnostic criterion it suffers from the disadvantage that it is often weak. Certain cultures produce only a narrow dextrin zone around the colony even when incubated for long periods.

Group III comprises two distinct types, some of the properties of which are shown in Table 4. Resistance to heat is greater in subgroup *B* than in any other type of micrococcus examined in this work. We have not explored the upper limit of their resistance beyond testing six cultures at 70° C. for 30 min. All six developed when the heated suspensions (in milk) were incubated, but none gave growth on the plates prepared immediately after the heating.

Table 4 shows that *B* may be distinguished from *A* by several other features, especially by a lower maximum temperature for growth, a slower and more restricted growth on artificial media, the action on milk and gelatin, and a greater range of fermentative powers. Every culture of *B* fermented glycerol and at least one of the pentoses. Raffinose is fermented by a considerable proportion of the cultures of *B* but by no other micrococcus or staphylococcus examined in this study. Pentose fermentation has likewise not been detected among the other strains, although every culture of group I was not tested for this property. Litmus milk affords a useful distinction between *A* and *B*. In *A* the majority of strains (the lactose fermenters) produce acid and reduction of the litmus and finally clotting, the changes generally starting in the surface layer. A yellowish growth commonly accumulates at the surface and also at the base of the tube. In *B* there is usually no change apart from a slow acid formation; exceptionally, clotting may occur after 10 or more days at 30° C.

A feature of subgroup *A* is the strong greenish yellow pigmentation of the growth on lemco agar. On agar + 20–30 % milk the colour is often less intense. The chromogenesis thus provides a useful distinction from group I. On other media the difference between the two groups may be less distinct. For example, on glucose agar and potato certain cultures of III *A* produce a thick rugose growth, and in some cases the wrinkles may acquire a colour approaching the orange-yellow typical of group I. In one variant culture of III *A* the growth is brownish, or brownish with lemon-yellow sectors, and at a later stage a purplish zone may appear near the margin. In subgroup *B* the intensity of the greenish yellow colour varies considerably, and five cultures have produced no detectable pigment. From one of the pigment-forming strains a non-chromogenic (but otherwise identical) variant was separated. The variant has now been propagated for over 5 years without the reappearance of colour. In both subgroups the growths on solid media may be friable, coherent or even viscous. These distinctions have proved to be unstable.

Table 4. *Characters of group III*

	A	*B*
No. of cultures	100	184
Growth on agar	Rapid, luxuriant greenish yellow	Slow, restricted, pale greenish yellow or unpigmented
Litmus milk	90 acid, generally clotted; 10 alkaline	Slowly acidified, rarely slowly clotted
Gelatin liquefaction (22° C. 4 weeks)	93 + 7 –	–
Acid from glycerol	–	+
Acid from xylose*	–	178 + 2 –
Acid from arabinose*	–	164 + 16 –
Acid from lactose	90 + 10 –	+
Acid from raffinose*	–	114 + 66 –
Casein digestion	11 + 89 –	–
Fat hydrolysis Tributyrin hydrolysis	1 + 99 –	–
Nitrate reduction	69 + 31 –	90 + 94 –
Growth with NH_3-N	69 + 31 –	119 + 65 –
Growth at 37° C.	+	– or slight
Survival at 65° C. 30 min.	– or small survival	+

* Four cultures of *B* were not tested.

Several investigators who utilized the liquefaction of gelatin as a differential criterion in *Micrococcus* incubated the gelatin cultures for extended periods. This practice would result in showing four liquefying strains in subgroup *B*. The periods (at 22° C.) required for the appearance of liquefaction were 6, 11 (in the case of two cultures) and 12 weeks.

The data obtained do not warrant any division of group III beyond the two subgroups defined in Table 4. Among the several variations noted in each subgroup, the only instance of an association of two characters is in subgroup *A* where nitrate reduction and the ability to grow with an ammonium salt as the only source of nitrogen are perfectly correlated. Hucker's (2, 3) classification of the micrococci, which is founded on pigment production, action on gelatin and nitrate, and ability to utilize ammonium salts, would distribute *A* and *B* among several species, some of which would include strains of both *A* and *B* and also organisms that we have placed in groups I and II.

Subgroups *A* and *B* appear to be sufficiently distinct from each other and sufficiently different from other micrococci to deserve recognition as two species. It is highly probable that each has in the past been placed in several different species, and their nomenclature is thus a complex question. Experience with the micrococci suggests that they could not

be identified with any species of which at least the pigment and the action on gelatin and milk are unrecorded. Taking this view of the minimum characterization which must apply to the majority of strains of each type, *A* conforms to *Micrococcus luteus* Cohn *emend.* Lehmann & Neumann (20) and *B* to *M. varians* (Dyar) Migula (30).

Although *M. luteus* has been regarded (Buchanan (31), Bergey (32)) as the type species of the genus, there is much uncertainty concerning its identity. Cohn's original characterization, which was published before the era of pure cultures, is totally inadequate, and the first amplification of the species diagnosis which could be accepted as defining a distinct type of micrococcus seems to be that of Lehmann & Neumann. Within the limits of the characters they use it fits the majority of strains in subgroup *A*. Of special significance are their descriptions of tetrad formation, growth at 37° C., pigmentation, liquefaction of gelatin and acidification and clotting of milk. At a later date Hucker (3) defined the species quite differently. According to his characterization there is no action on gelatin, milk or lactose and acid is formed from mannitol. These characters have been incorporated in the description of *M. luteus* in the later editions of *Bergey's Manual* (32). Since Hucker's type ferments mannitol and forms ammonia from peptone and fails to attack starch it probably represents one of the numerous variations occurring in our group I. If the name *M. luteus* can be retained without becoming a permanent source of confusion, there is little reason for the rejection of Lehmann & Neumann's definition. The characters of group III *A* described here provide some additional detail.

Migula's characterization of *M. varians* indicates that the growth on potato is more profuse than that typical of subgroup *B*, but in other respects (tetrad formation, pigmentation, inability to liquefy gelatin, acid production from lactose and weak acidification of milk) it fits this type of organism. Conn's (33) *M. varians-lactis* is clearly a different species.

Hammer & Trout (29) have previously identified as *M. luteus* and *M. varians* two of the three main types of thermoduric micrococci isolated by them from dairy products. Their brief descriptions indicate, however, that they probably used the name *M. luteus* for type *B* and *M. varians* for type *A*.

A micrococcus which differs from every strain so far discussed was isolated on one occasion; four colonies of the organism were detected on plates prepared from a sample of pasteurized milk. It withstands heating at 63° C. and in its cultural reactions it approaches group III *A*. Its more distinctive features are: the cells are easily decolorized when stained by Gram's method; the viability on agar is poor; the growth on solid media is translucent and may acquire a faint pink or straw colour; lactose is not fermented and there is no action on milk. The status of this organism is uncertain; in the meantime it will be considered to be a variant of III *A*.

Discussion

In this study of representatives of the *Staphylococcus-Micrococcus* complex occurring in milk, an attempt has been made to discover properties of the organisms that will provide the basis of an orderly classification. There appear to be definite advantages in attributing importance to those properties that arrange the strains in a virtually continuous series. This pattern seems to reflect the general character of the organisms, and at the same time it is closely linked with their ecology. If our suggestion is well founded a practical problem is to define the more important parts of the series. The intermediate group

recognized in this paper, i.e. the organisms that do not ferment sugars, may not be homogeneous and little is known about the stability of characters on which it is based. Nevertheless, it is demarcated fairly distinctly from the other two groups. Only three cultures were found to be frank connecting links. They are named strains which have been propagated artificially for long periods. One, *Staphylococcus cremoris-viscosi* Hammer & Cordes (34), N.C.T.C. 963, appears to be an extreme form of group I*B* which, on account of its aerobic nature, its slow and feeble acid formation from glucose and its sensitivity to salt, stands on the border of group II. A culture received as *Micrococcus varians* (N.C.T.C. 2685) and another as *M. conglomeratus* (N.C.T.C. 2677) are almost perfect intermediates between groups II and III and they cannot satisfactorily be placed in either. Whether these strains are in their original condition or have changed during artificial culture cannot be decided. No matter where lines of division are drawn intermediate strains are perhaps inevitable. A clear distinction between groups I and III appears to be the essential, and the most suitable means of providing it seems to be the recognition of an intermediate group.

The grouping here suggested is intended to apply primarily to the forms that occur commonly and numerously in milk. To what extent it can be utilized for those derived from other sources is uncertain, but descriptions of the multitude of named species indicate that a considerable proportion is represented in our series of cultures. The genus *Micrococcus* provides what is probably the worst example in bacteriology of uncritical systematic work. Already in 1900 Migula (30) was able to give descriptions of over 200 species. Air, a source from which various species of *Staphylococcus* and *Micrococcus* have been obtained, commonly carries organisms that may be allotted to our group I. This suggests that the human or animal body may be an important source of the air cocci. Little is known with certainty about the distribution of micrococci in nature. Reports of their isolation from water, soil and other materials do not always exclude the possibility that the organisms were merely contaminants derived from laboratory air.

SUMMARY

A study of 799 cultures of staphylococci and micrococci, isolated mainly from milk, showed that according to their general character the strains could be arranged in a virtually continuous series. At one extreme is the pathogenic staphylococcus and at the other a thermoduric saprophyte. The series consists of three main groups:

(1) The staphylococcus group in which the organisms are sugar fermenters and relatively sensitive to heat. They are, mainly at least, parasites of the animal body. Four subgroups are distinguishable. In three ammonia is formed from arginine, and in two acetoin is formed from glucose. A test for acetoin production defines those mannitol fermenters which do not produce coagulase.

(2) An intermediate group in which the organisms are obligate aerobes and do not produce acid from sugars.

(3) The dairy micrococci, a group of thermoduric sugar fermenters which occur frequently on dairy equipment and in pasteurized milk. The group comprises two species conforming to *Micrococcus luteus* Cohn *emend.* Lehmann & Neumann, and *M. varians* (Dyar) Migula.

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364. STUDIES ON THE BACTERIOLOGICAL FLORA AND KEEPING QUALITY OF PASTEURIZED LIQUID CREAM

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The sale of cream has been prohibited in Britain since 1940 owing to wartime milk shortages, but prior to that date cream production was an important activity of the dairy industry.† Considerable quantities of clotted cream were produced in south-western England, whilst tinned sterilized cream (of which a proportion was imported) also enjoyed an increasing demand. The bacteriology of the former product has been discussed by Crossley (1) and that of the latter by Nichols (2). The bulk of the cream supplies, however, consisted of 'fresh cream', i.e. pasteurized liquid cream, and since little information concerning the bacteriological aspects of this type of cream has been published, some data accumulated prior to 1939 may be of interest.

A general account of the cream trade in Britain was published in 1932 by the Ministry of Agriculture and Fisheries (3). The trade conditions are unusually difficult and some features which have an important bearing upon bacteriological requirements may be outlined here. The farmer-retailer is usually unable to meet the special conditions of the trade and the main output of cream comes from dairy factories. Owing to the lower cost of transporting cream as compared with the equivalent milk, cream separation in the consuming centres is not usually favoured. Separation is mainly carried out at country milk-collecting depots, where facilities are also available for utilizing the separated milk.

Immediately after separation the cream is subjected to some form of heat treatment and cooled to 40° F., or lower. It is then distributed in bulk, either (i) in non-returnable cans (with detachable lids) of ½–2 gal. capacity direct to small retail dairymen, or (ii) in churns via wholesale town depots. A second heat treatment may be carried out at wholesale depots, after which the cream is packed into non-returnable bulk cans or (less commonly) directly into small retail containers. Large quantities of cream are retailed by small dairymen who purchase their supply in bulk (often from distant sources) and pack it into small retail containers—usually single-service waxed paper cartons. Such dairymen rarely possess processing equipment and may even lack cold-storage facilities; moreover, the method of filling cartons, which sometimes merely comprises dipping cream from an open bowl standing on a shop counter, may itself be a means of increasing bacteriological contamination.

Owing to various factors the demand for cream is very irregular and often unpredictable. There is, however, a regular marked increase in sales at the week-end, when about 70% of the weekly sales take place. In addition there is a very pronounced seasonal increase

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† The annual cream output represented a liquid milk equivalent of 71·6 million gallons in 1939, whilst large additional quantities were imported from Ireland. Of this total 12·2 million gallons were utilized for manufacture of sterilized cream and 59·4 million gallons for 'fresh cream'. Since cream is a valuable food and in Britain its production may offer a more profitable outlet for surplus milk supplies than does cheese or butter manufacture, it may be anticipated that this branch of dairying will assume considerable importance when normal conditions return.

during June and July and at Christmas. These fluctuations are met in part by other forms of milk utilization at the large separating depots, but some bulk cold storage of the cream (usually in churns) is frequently necessary at either the country creamery or the town wholesale depot. The seasonal peak demands may also necessitate importation of additional supplies from Ireland. Under other circumstances it may be necessary to utilize cream surplus to market requirements; such disposal may be achieved at the country depot by making butter but may present a more difficult problem to the town wholesaler or retailer. The factors responsible for variations in the demand for cream have been discussed by Bransby (4).

Owing to the general structure of the trade, keeping quality is of major importance since, in addition to any storage required to meet fluctuations in demand, the cream may have to be transported over long distances in comparatively small bulk containers, at high atmospheric temperatures and without any serious attempt to secure refrigeration of the rail or road vehicle.

Furthermore, various hazards may arise from multiple handling during wholesale and retail distribution, whilst the domestic consumer may expect the cream to remain sweet up to 24 hr. after purchase, again at atmospheric temperature. In practice, therefore, success depends upon the efficiency of processing at the original separating and pasteurizing depot, and the cream when dispatched should contain very few organisms in order to offset the adverse factors which may arise during transport and distribution.

Objects of the investigation

Bacteriological control of cream processing has been studied over a period of 10 years at a large country depot engaged in cream distribution on a nation-wide basis. The investigations were undertaken to establish the sources of bacteriological contamination and to devise suitable means of reducing infection to a minimum. During this work it became apparent that a simple routine control test was required to forecast the probable keeping quality of pasteurized cream, which could be applied to large numbers of units such as individual churns. Experimental work was undertaken with this object in view and a rough control test was evolved.

Rapid estimation of keeping quality

Preliminary work

Trade requirements could be met by a keeping quality at atmospheric temperature of about $2\frac{1}{2}$–3 days from the time of processing, but a high output was required during the summer months when mean atmospheric temperatures exceeded 65° F. In terms of the plate colony count this involved the production of cream containing not more than 5000 bacteria per ml. and preferably only a few hundred per ml. Acid-producing cocci and coliform bacteria appeared to be the most significant contaminants and souring was the usual form of deterioration. Coliforms caused particularly rapid souring at summer temperatures; the cocci were less rapid acid producers but seemed able to develop over a very wide range of temperatures and eventually caused souring even at 40° F. The development of a bitter flavour unaccompanied by any definite odour or visible change was also not uncommon, and usually seemed to be associated with extensive proliferation of aerobic spore-forming bacilli. The bitter flavour was doubtless due to proteolytic

changes and later the cream became putrid. This defect occurred most frequently when cream of very low initial bacterial content was exposed to high atmospheric temperatures.

When large numbers of cream samples were considered, a general relationship was observed between the colony count and keeping quality, but study of individual records showed that on many occasions this correlation failed to apply to individual samples. Consequently the colony count could not be used as an index of keeping quality and in any case the time, labour and materials required precluded its use on an extensive scale. Similarly, methylene-blue reduction tests appeared to be of uncertain value and yielded very inconvenient reduction times for practical control purposes. The presence of coliform organisms markedly reduced keeping quality and the effect was significant even when the total colony count was very low. However, the coliform test, like the colony count, required a minimum period of 2 days to complete and moreover a large proportion of samples contained no coliform organisms.

Acidity tests are in common use, but can only reveal deterioration which has already taken place. It was found that acidity tests applied to fresh cream immediately after processing gave little indication of potential keeping quality and a rise in acidity at this time was only indicative of some gross failure. Such tests were sometimes a useful means of grading cream as received at wholesale depots after a long journey, although obviously it would have been preferable to apply control measures at an earlier stage.

It was felt that a suitable test should comprise a reasonably rapid estimation of the rate at which a sample of cream (initially of normal acidity) will develop acidity when exposed to atmospheric temperatures, and should be applicable to the individual bulk units—normally churns—in which cream is stored after processing. Any unit of doubtful potential keeping quality could then be detected before dispatch and diverted for butter-making whilst still in good condition. A period of 24 hr. was available for completion of the grading test. (The basis of this time period is discussed on p. 266.)

The basis of the method studied was the inoculation of cream into sterile milk, followed by observations of acidity development using a visual indicator in place of normal titration methods. Preliminary experiments were undertaken to determine the size of cream inoculum required to produce suitable acidity changes within a convenient time period, the most desirable incubation temperature, and a suitable indicator. The basis of comparison was the actual keeping quality time at atmospheric temperature, as determined by regular tasting carried out by a panel of three tasters. In order to simulate commercial conditions the keeping-quality tests were carried out over the range of atmospheric temperatures prevailing at different seasons and the work was spread over several years. This method was adopted in preference to keeping-quality tests performed at a constant temperature such as 18 or 20° C., since trials of this method sometimes yielded results which differed considerably from those obtained by storage at atmospheric temperature. The details of this work need not be elaborated here and the grading test which was eventually adopted will be described.

TWENTY-FOUR-HOUR GRADING TEST

(i) *Test medium.* Separated milk containing 0·01% bromcresol purple (standard 1% solution prepared by dissolving 1 g. indicator in 19 ml. decinormal sodium hydroxide and making up to 100 ml. with distilled water). Fill out 10 ml. quantities into sterile plugged test-tubes and sterilize by steaming for 20 min. on four successive days.

(ii) *Sampling.* Sterilized plunger-dippers are used for obtaining cream samples from churns or other containers; the samples are taken into sterilized sample bottles, which should be of 3–4 oz. capacity and preferably a wide-mouth type fitted with a screw-cap closure. The cream should be thoroughly mixed by plunging and the usual aseptic precautions should be observed during sampling.

(iii) *Laboratory testing.* From each sample 1 ml. of cream is inoculated into 10 ml. of milk plus indicator, using a fresh sterile pipette for each sample and employing normal aseptic methods during inoculation. The inoculated tubes are then incubated at $30 \pm 1°$ C., and inspected after 16–17 hr. and again after 24–25 hr.

(iv) *Examination of tubes.* The following notation is used for recording observations:

− No visible changes.
\+ Slight acidity, indicated by faint yellow mottling.
\+ + Definite acidity, shown by an even bright yellow colour, but no clot.
\+ + + Acid and clot.

Other signs may be used if desired to record changes such as gas production or peptonization. Very occasionally tubes may develop an alkaline reaction, denoted by a deep purple colour.

(v) *Interpretation of results.* Four grades are differentiated:

	Observation	
Grade	16 hr.	24 hr.
1	− or +	− or +
2	− or +	+ +
3	− or +	+ + +
4	+ + or + + +	+ + +

Application of the grading test

It was found that two routine testers could easily grade up to 60 individual churns of cream per day; most of the time and labour involved was absorbed in sampling. Churns of cream were cold-stored for 24 hr. until their grading was known, except that the lowest grade cream was detected in 16 hr. Trade experience showed that in general cream placed in the first two grades could safely be dispatched for sale as liquid cream, whilst cream graded 3 or 4 could either be subjected to a second heat treatment or churned into butter.

It may be mentioned here that the lowest grade cream usually yielded first quality butter; the bacteriological standards required for the cream trade seemed to be much higher than was necessary for successful butter-making.

Data obtained from examination of 2558 samples of heat-treated cream are summarized in Table 1 A and show the general relationships between the grading test, colony count, coliform test and keeping quality at atmospheric temperature. The decline from grade 1 to grade 4 was accompanied by a pronounced reduction in the mean keeping quality, amounting to a difference of roughly 10 hr. between each grade. The mean colony counts (on standard yeastrel milk agar at 37° C.) showed a progressive increase from grades 1 to 4 and also a general correlation with mean keeping quality. However, the range of counts was relatively narrow and the figures illustrate the difficulty of grading on the basis of colony counts when the total number of organisms is small; this point is particularly illustrated by comparison of grades 2 and 3. The coliform-test results were probably more significant and showed a marked rise in the incidence of positive tests between each grade. There was therefore an undoubted general relationship between

coliform contamination and reduced keeping quality, whilst the records of individual samples showed that even a small infection (e.g. present only in 10 or 1 ml. of cream) resulted in shorter keeping quality; the elimination of coliform contamination is clearly of great commercial importance. Nevertheless, even if it were practicable, the coliform test alone would not provide an entirely satisfactory grading test, since the data show that 15% of the grade 4 samples were free from coliform organisms.

A standard incubation temperature of 30° C. appeared to be most satisfactory for general use and probably provided optimum conditions for most of the organisms present in cream. The grading was possibly a little severe during cold weather in that grade 3 might then possess adequate keeping quality for commercial distribution. On the other hand, at summer temperatures exceeding 70° F. no grade of heat-treated cream could be relied upon to possess sufficient keeping quality in the absence of refrigerated or cool conditions of transport and storage.

Table 1A. *Relationships between rapid grading test, bacteriological tests and keeping quality*

			Coliform positives		Keeping quality by taste at atmospheric temperature (shade)	
No. of tests	Grade	Geometric mean colony count per ml.	No.	%	No. of tests	Mean keeping quality in hr.
1773	1	180	141	7·9	1078	66·8
324	2	1,370	121	37·3	510	55·2
240	3	2,430	123	51·2	733	46·9
221	4	14,030	188	85·1	369	35·0
2558					2690	

Table 1B. *Summer and winter test results for all grade 1 samples*

808	1 (summer)	150	62	7·8	612	54·7
965	1 (winter)	220	79	8·2	466	82·7
1773					1078	

The importance of atmospheric temperature conditions is illustrated by the data in Table 1B, which were obtained by splitting all the grade 1 samples into summer (April–September) and winter (October–March) groups. Although the mean colony counts and coliform positives varied little between the two groups (indicating a uniform standard after processing) the mean keeping quality times differed by 28 hr.

It has been mentioned (p. 262) that deterioration sometimes appeared as a bitter flavour. Samples which developed bitterness were mainly those which had shown no change in the grading test, and at high atmospheric temperatures of storage (exceeding 65° F.) grade 1 samples commonly became bitter. Almost invariably such samples had low colony counts and were free from coliform organisms immediately after processing; the flora showed a marked predominance of aerobic spore-forming organisms. Unsuccessful attempts were made to devise a rapid means of detecting potentially bitter samples. In a few samples alkalinity and peptonization were obtained in the milk tubes but no reliable means was found of ensuring this reaction within a 24 hr. incubation period. In practice, however, bitterness was less important than souring, since ample time was usually available for distribution before bitterness developed and the defect was mainly associated with very high atmospheric temperatures.

The bacteriological control of cream processing

Methods of processing

Processing may be divided into three stages, viz. heat treatment, cooling and ageing. Heat-treated cream should contain smaller numbers of organisms than would be permissible in pasteurized milk; the particular processes and plant employed are subject to variation but may be classed as either high temperature-short time or holding methods.

The H.T.S.T. method is more common in Britain and facilitates rapid, continuous handling from the separator onwards. Older plants may comprise a 'flash scalder' or tubular heater followed by an open capillary type cooler, but the most popular plant is an internal plate-type heat exchanger similar to that employed for the heat treatment of milk, wherein both heating and cooling are performed in continuous flow within one machine. The cream is usually heated at 180–190° F. for 10–20 sec. and cooled to 40° F. or lower. A second heat treatment may be given after cold storage of the cream for 24–48 hr.; in any case double heat treatment is common, the first at the separating depot and the second at the wholesale distributing depot.

The low-temperature holding process comprises batch heating, holding and cooling in some form of heater-holder vat. Holding at 145–155° F. for 30–60 min. is usual, whilst both heating up to the holding temperature and subsequent cooling to 40° F. are comparatively slow processes. In some cases rapid cooling (to secure increased output) is adopted by pumping the hot cream over an independent capillary type cooler.

Subsequent to processing the cream is usually subjected to further cold storage (ageing) at 34–36° F., to obtain increased viscosity; the storage period varies from 12 hr. to several days. Practically all the increase in viscosity occurs during the first 24 hr. but longer periods may be utilized to balance fluctuating sales demands. Special refrigerated vats may be employed but in Britain cream is usually held in churns during ageing.

Commercial properties

Apart from considerations of keeping quality, flavour and viscosity are important commercial properties which influence the choice of processing methods.

Loss of natural cream flavour and the development of cooked flavour is least pronounced when low-temperature holding is employed. Short-time heating at 180° F. (particularly if performed twice) results in some cooked flavour, but the change is not pronounced and such cream is generally acceptable.

Viscosity is directly dependent upon fat content and is artificially increased by homogenization when the fat content is 40% or less. In addition, it is influenced appreciably by the heat treatment employed and by the rate of cooling. Low-temperature heating, followed by slow, gentle cooling, attains the highest viscosity for a given fat content. The combined effects of high-temperature heating, rapid cooling, and violent agitation associated with the H.T.S.T. process normally result in rather lower viscosity, but in cream of 50% fat content the loss of body is not serious unless the cream is heated more than once. In any case, ageing for 24 hr. is highly desirable in the control of viscosity and this requirement fixes the time period available for completion of a rapid control test.

Table 2. *Bacteriological examinations of plant samples*

Series	Stage of processing	No. of samples	Colony count per ml.			Coliform organisms						Keeping-quality grading test									
			Geometric mean	% counts		Absent		Pos. 1 ml.		Pos. <1 ml.		1		2		3			4		
				Under 1,000	Under 10,000	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%		No.	%	
A	Discharge from cooling section of H.T.S.T. plant. First heat treatment	181	2,180	33·7	82·9	164	90·6	4	2·2	13	7·2	121	66·9	33	18·2	6	3·3	14·9%	21	11·6	
B	As series A, but after a second heat treatment	382	180	83·8	97·4	369	96·6	5	1·3	8	2·1	334	87·4	28	7·3	12	3·1	5·2%	8	2·1	
C	Cream reprocessed following a long journey	71	10,000	18·3	45·1	61	85·9	4	5·6	6	8·4	19	26·8	20	28·1	13	18·3	45·1%	19	26·8	
D	Two gal. cans filled directly from H.T.S.T. plant after second heat treatment	206	100	89·8	99·0	195	94·7	7	3·4	4	1·9	181	87·9	13	6·3	9	4·4	5·8%	3	1·4	
E	Churns sampled after 24 hr. cold storage at 35° F.	902	360	75·7	91·4	738	81·8	55	6·1	109	12·1	649	71·9	135	15·0	71	7·9	13·1%	47	5·2	
F	Samples from can or carton filler, cream heated twice by H.T.S.T. process	521	1,170	52·4	81·8	357	68·5	55	10·6	109	20·9	342	65·6	91	17·5	44	8·4	16·8%	44	8·4	
G	Cream treated in sealed containers by holder process	74	3,890	32·4	63·5	72	97·3	2	2·7	—	—	61	82·4	8	10·8	2	2·7	6·8%	3	4·1	
	Total	2,337																			

Sources of bacteriological contamination

(*a*) *Raw milk supply*

It has been shown by Sargeant *et al.* (5) that cream separated from designated milk of low bacterial content possessed a superior keeping quality (whether raw or heat-treated) to that of cream derived from ordinary mixed milk. At large depots daily selection of individual milk supplies can be practised only to a limited extent and reliance must be placed upon regular control tests of the supply as a whole. Nevertheless, it is important to avoid delayed separation after receipt of the milk or the holding of the raw cream at separating temperature (about 110° F.) for long periods prior to heat treatment, since both procedures encourage undesirable bacterial proliferation. It has been found desirable to adopt a continuous process whereby receipt of the milk is followed at once by separation and heat treatment of the cream; the data which follow were obtained when using this procedure with a commercial milk supply of average quality.

(*b*) *Heat treatment*

Given prompt milk handling and separation, the cream as discharged from the heat-treatment plant normally contained few organisms, particularly when the H.T.S.T. process was used at a high operating temperature. Thus a single high-temperature heat treatment (Table 2, series A) yielded cream containing an average of only 2180 bacteria per ml., and 83% of samples contained less than 10,000/ml., but 9·4% of samples contained coliform organisms and rapid grading tests placed 14·9% of samples in the doubtful category (grades 3 and 4). When a second heat treatment was given following a 24 hr. ageing period (series B), the average colony count was reduced to 180/ml., whilst counts below 10,000 comprised 97·4% of the total and 83·8% were below 1000/ml. The incidence of coliform positives fell to 3·4% and only 5·2% of samples gave doubtful grading results. The double heat treatment was therefore a considerable advantage.

In contrast, if the period between two heat treatments comprised a long journey at atmospheric temperature between depots (series C), very different results were obtained. Although the second heat treatment was efficiently performed, the cream contained an average of 10,000 bacteria per ml., 14% of samples were coliform positive and 45% fell within the unsatisfactory keeping quality grades. Thus a heat treatment which normally was effective could not be relied upon to offset the effect of extensive bacterial proliferation prior to processing.

The small proportion (about 5%) of unsatisfactory samples taken direct from the heat-treatment plant was due mainly to contamination of the cooling section arising from either inadequate sterilization or reinfection by passage of the first flow of cream at too low a temperature through the heating section. In addition, occasional contamination was derived from rubber-jointing gaskets between the plates of heat exchangers used in this investigation; such gaskets eventually developed a porous, spongy condition in which cream residues were retained. Prompt removal of worn rubbers obviated this source of contamination. In general the difficulties are avoidable and with rare exceptions cream delivered by the heat-treatment plant should be satisfactory.

(*c*) *Post-heating contamination*

In commercial practice contamination was associated mainly with the series of processes involved in filling the cream into containers and in general handling during

distribution. It was sometimes possible to fill the cream directly from the cooling-section discharge into the small cans used for wholesale distribution, and thus minimize the risk of post-processing contamination. (Efficient steam sterilization of the cans was of course essential.) Data from 206 such samples (Table 2, series D) illustrate the efficacy of this procedure; samples from the cans yielded results which were almost identical with those obtained from direct plant samples (series B) and the proportion of low-grade samples remained at 5·8%.

Ageing and storing a large bulk of cream packed in small containers may be impossible owing to limitations of cold-storage space and usually some cream must be stored in churns. Although during this investigation the churns examined by a rinse technique appeared to contain few organisms, the heat-treated cream also contained few organisms and the additional contamination derived from churns was sometimes of importance. Further spasmodic contamination probably resulted from direct manual contact with the churn rim and upper internal surfaces, since considerable handling was involved in filling the churns and placing them in cold storage. Moreover, cream inevitably contacted the churn rim when removed (after ageing) into other containers. A series of 902 cream samples (Table 2, series E) was taken from churns held for 24 hr. in cold storage. In comparison with direct plant samples (series B) the mean colony count of this series showed only a slight increase to 360/ml. and 75% of samples gave counts below 1000/ml.; the plate count therefore revealed little additional contamination. However, the proportion of coliform negative samples dropped to 82% and positives in less than 1 ml. of cream showed a marked rise from 2 to 12%.

The grading tests also revealed a significant rise (to 13%) in the proportion of low-grade samples.

Occasionally large refrigerated cream-storage vats of 250 gal. capacity, fitted with slow-moving swinging agitators, were used instead of churns for ageing the processed cream. A series of ten comparisons between cream as discharged from the H.T.S.T. plant after a single treatment (similar to Table 2, series A) and cream aged for 24 hr. at 40–45° F. in the storage vats yielded the following figures:

	Discharge from H.T.S.T. plant	Storage vat
Mean colony count 37° C.	5,070	22,000
Coliform organisms absent	8 samples	6 samples
Coliform organisms present 1 ml. cream	2 samples	Nil
Coliform organisms present <1 ml. cream	Nil	4 samples

Although the number of tests was small it seemed clear that in respect of bacteriological contamination this process might be no better than churn storage. Adequate sterilization of large vats of this type by means of steam or hot water was not easy; the use of hypochlorite solutions would have reduced the difficulties but at the time this procedure was illegal. Other possibilities of infection arose from the additional pumps and pipelines required in conjunction with the vats. In addition to the possibility of plant contamination, the temperature of storage seemed liable to be rather higher than that obtainable by using churns held in cold storage. Nevertheless, the process possessed considerable operating advantages and no doubt could be rendered bacteriologically satisfactory with the aid of hypochlorite sterilization.

The final creamery operation comprises filling the cream into the containers in which it is distributed—either cans (wholesale) or cartons (retail). Some contamination from filling

plant is inevitable and may be considerable unless stringent precautions are observed. Taps and valves (especially if not of drip-proof design) may be a serious source of infection; it should be possible completely to dismantle the plant and subject all the individual parts to cleaning and steam sterilization. In addition, care is needed to avoid manual handling of surfaces which contact the cream, e.g. the internal surfaces of filler-bowls, taps, valves, containers or their closures. Data from a series of 521 samples (Table 2, series F) taken at a filling plant clearly illustrate the significance of this operation when compared with those from samples taken at earlier stages (series A–E). Thus the plate-colony count, though still low, revealed a definite increase. The coliform tests yielded even more significant results, since the proportion of negative tests fell to 68·5% as compared with 81·8% for series E and 96·6% for series B, whilst that of tests positive in less than 1 ml. rose to 20·9%. The practical significance of these changes was revealed by an increase (to 16·8%) in the proportion of samples of doubtful keeping quality.

The H.T.S.T. method of processing was also investigated, in rather less detail, at another plant which comprised a tubular-type heater and open capillary-type cooler. The cream as discharged from the heater was satisfactory, but considerable contamination was derived from the cooler in spite of prolonged efforts to secure sterilization of the surface by means of hot-water rinses (hypochlorite sterilization was illegal at the time). Of 335 samples taken from the cooler, 66 (19·7%) contained coliform organisms; the plant was obviously inferior to the enclosed plate-type heater and cooler.

Very similar results were obtained from a cream-pasteurizing vat (employing a holding process at 145° F. for 60 min.) and open capillary-type cooler. In this case 48 samples taken from the holder discharge were all coliform negative, but 73 samples taken from the cooler yielded 14 (19·2%) coliform positives.

In addition, the possibility of contamination during ageing and during filling of containers was common to all the foregoing processes. It appeared, therefore, that the best method of eliminating contamination during cooling, handling after processing, and from the container itself, would be to heat and cool the cream in the sealed container in which it was to be dispatched. A small-scale commercial process was developed to investigate this method and yielded very promising results until the work was discontinued on the outbreak of war. The cream was rapidly 'flash' heated to 150° F. and filled whilst hot into 2 gal. cans, which were sealed immediately and then held at 150° F. for 60 min. by immersion in a water-bath and subsequently cooled slowly in running water. In preliminary trials the cream was also heated up to holding temperature in the small containers, but no advantage appeared to be derived from this lengthy process.

Data relating to 74 samples are given in Table 2, series G. It will be observed that higher colony counts (mean 3890/ml.) were obtained than in the case of H.T.S.T. treatment; the increase was most noticeable during the summer months. The incidence of presumptive coliform positives was, however, the lowest of any series; all samples were, in fact, coliform negative since the two positive reactions obtained were actually 'false positives'. The grading tests also yielded highly satisfactory results, which indicated that the keeping quality of the samples was similar to that of samples submitted to a double H.T.S.T. process, but without the subsequent deleterious effects arising from contamination associated with ageing in churns and filling of containers. The laboratory findings were confirmed in commercial experience. The increased number of organisms which survived low-temperature holding was apparently of little importance, probably because the

additional flora largely consisted of thermoduric micrococci and streptococci similar to the types found in pasteurized milk; it is known that such organisms have no rapid effect upon the keeping quality of milk. The nature of the flora probably accounted also for the absence of bacteriological difficulties associated with the slow-cooling process. The thermoduric flora of the raw milk supply was probably an important and sometimes variable factor, since some individual samples of holder-process cream yielded extremely low-colony counts.

As would be expected, low-temperature holding and slow cooling produced cream of excellent flavour and viscosity; indeed the 'body' of 50–53% fat-content cream was sometimes excessive and the process seemed ideally suited to cream of 40% fat content. Large-scale development of this particular process remains a mechanical problem for the future, and unfortunately it is probably impracticable for large consignments dispatched in churns.

Detailed investigations of the coliform flora of cream have been described in an earlier paper (6) and it is only necessary to repeat here that the distribution of coliform strains differed considerably from that found in either raw or pasteurized milk. The variable nature of the coliform contamination arising from handling and packing operations resulted in a rather generalized distribution of coliform strains. Thus the *aerogenes-cloacae* group was nearly as numerous as the *coli* group and 'irregular' strains were more frequent than in other dairy products. Heat-resistant coliform strains appeared to be less common in cream than in pasteurized milk. Finally, it may be observed that 'false-positive' presumptive coliform reactions were not uncommon, particularly with holder-process samples.

(*d*) *Influence of cold storage*

The functions of cold storage in the handling of cream have already been indicated, and in practice storage periods up to 7 days are by no means uncommon. General trade experience seems to indicate that cold storage for several days has no adverse effect upon the keeping quality of cream when subsequently exposed to atmospheric temperature, provided it is of good bacteriological quality. A few laboratory investigations seemed to confirm the trade belief.

On three occasions a churn of cream was held in cold storage at 35–37° F. and sampled daily. In addition to bacteriological and grading tests the samples were held constantly at 20° C. after removal from cold storage and submitted to tasting tests until no longer acceptable. The cream was treated by a double H.T.S.T. process and on two occasions it happened to contain very few organisms, whilst on the third occasion it was unsatisfactory. The results (Table 3) revealed no definite changes during cold storage and the small differences were probably due to sampling and experimental errors, but tests of a larger number of samples would have been desirable.

A rather more extensive series of samples was examined in order to determine the changes in the plate-colony count during cold storage up to 4 days. The trials covered both the double H.T.S.T. process (cream stored in churns) and the holder process (cream stored in 2 gal. cans). All the holder-process trials were, however, carried out during the winter months when the bacterial content of this type of cream was at a minimum. The mean results (Table 4) seemed to indicate that the colony count very slowly increased, but that no serious objection could be raised to cold storage up to 3 or possibly 4 days.

Obviously, however, these preliminary observations should be confirmed by a systematic study of the cold storage of cream.

Table 3. *Influence of cold storage at* 35–37° F. *on cream quality*

Trial	Test	Cold-storage period (days) prior to testing: 1	2	3	4	5	7	8	11
1	Colony count	70	70	20	30	.	30	.	.
	Coliform test	–	–	–	–	.	–	.	.
	Grading-test category	1	1	1	1	.	1	.	.
	Keeping quality (hr.) at 20° C.	56	56	56	56	.	56	.	.
2	Colony count	300	460	440	.	.	.	.	.
	Coliform test	–	+1 ml.	+1 ml.	.	.	.	.	.
	Grading-test category	4	4	4	.	.	.	.	.
	Keeping quality (hr.) at 20° C.	30	30	30	.	.	.	.	.
3	Colony count	20	20	. .	60	20	.	20	30
	Coliform test	–	–	.	–	–	.	–	–
	Grading-test category	1	1	.	1	1	.	1	1
	Keeping quality (hr.) at 20° C.	64	56	.	56	56	.	64	64

Table 4. *Geometric mean colony counts of cream held in cold storage at* 35–37° F.

Process	No. of experiments	Cold-storage period (days) prior to testing: Nil	1	2	3	4
H.T.S.T. (double treatment)	16	1640	1620	1690	2260	4000
Holder (in sealed container)	17	120	210	390	330	410

(e) *Influence of atmospheric temperatures during distribution*

The distribution of cream (see p. 261) may involve exposure of comparatively small containers (e.g. 1–2 gal. cans) to atmospheric temperatures for considerable periods after dispatch from the processing depot. Whether distribution takes place between primary processor and retail distributor direct, or via an intermediate wholesale depot, the aggregate exposure to atmospheric temperature before the cream is purchased by the consumer may frequently be 24 hr. or longer. The changes which may take place in cream held at atmospheric temperature are therefore of considerable practical importance.

A series of experiments was undertaken in which samples of cream were examined after storage at atmospheric temperature for 24 hr.; the samples were held in sterile glass jars of about ¼ pint capacity. Six comparative trials were made wherein cream was sampled directly from an H.T.S.T. plant discharge (as in Table 2, series B) and again from the final filling plant after ageing in churns, the same cream being sampled as nearly as possible. The 12 samples were examined immediately after collection and again after storage at summer atmospheric temperature. The following figures were obtained:

Sample	Age when tested (hr.)	Mean colony count (per ml.)	No. of coliform positives	Grading-test no. in grade: 1	2	3	4
6 samples direct from H.T.S.T. plant	0	120	Nil	6	.	.	.
	24	2,140	Nil	5	.	.	1
6 samples direct from filling plant	0	300	1	5	1	.	.
	24	172,000	3	1	.	3	2

The direct-plant samples showed only a comparatively small increase in the colony count and were still coliform negative after 24 hr. storage; one sample reached the lowest category in the grading test, but five samples remained in the first grade. The filling-plant samples, when tested immediately, were apparently only slightly inferior to the direct-plant samples except for one coliform positive reaction (in 1 ml. of cream). Indeed, the

colony counts were very low and probably rather better than the average for such samples (cp. Table 2, series F). After holding the cream for 24 hr. at atmospheric temperature, however, the mean colony count rose to a high figure, three samples gave positive coliform reactions, and five samples were placed in the low-keeping quality categories of the rapid grading test. These results suggest two important points, viz. (*a*) the organisms present in direct-plant samples, being mainly spore-forming types, tend to multiply rather slowly at atmospheric temperatures, and (*b*) the miscellaneous contamination associated with later stages may introduce much more active types which may impair keeping quality significantly in 24 hr. despite an extremely small initial infection.

The above samples represent the contrast between initial and final stages, i.e. the sum total of bacterial infection at the processing depot. A more extensive series was examined wherein cream samples taken from the H.T.S.T. plant (second heat treatment), and from 2 gal. cans filled directly from the H.T.S.T. plant, were tested after ageing for 24 hr. at atmospheric temperature (Table 5). This work was also extended to include cream processed in a sealed container by the experimental holder method.

Previous work having shown (Table 2, series D) that post-heating contamination could be minimized by filling the cream cans directly from the cooling section of the H.T.S.T. processing plant, it was of interest to determine the effect of storage at atmospheric temperature upon cream handled under the most favourable conditions; similar considerations led to the choice of holder-process samples.

The results obtained from 180 direct-plant samples, when compared with those from similar samples (Table 2, series B) tested without ageing, indicated little deterioration of the cream. Thus the mean colony counts of the two series were 180 and 890/ml., the incidence of coliform positives showed no change, whilst the proportion of unsatisfactory grading tests increased from 5·2 to 8·9%. With 194 can samples, similar comparisons with original samples (Table 2, series D) revealed an increase from 100 to 3550/ml., in the mean colony count, coliform positive samples increased from 5·3 to 20·6%, whilst a pronounced increase from 5·8 to 49·5% occurred in the proportion of unsatisfactory grading tests. Clearly, therefore, the very small contamination of the original cream had increased to very significant proportions after exposure of the cream to atmospheric temperature; in this respect the findings resembled the well-known effects of post-pasteurization contamination upon the keeping quality of pasteurized milk.

The 72 samples of holder-process cream tested after ageing compared very favourably with samples tested immediately after processing (Table 2, series G). The mean colony count increased from 3890 to 5990/ml., the incidence of positive coliform tests (actually 'false positives') remained unchanged, whilst unsatisfactory grading results increased only from 6·8 to 8·3%. These data again indicated that it was possible completely to eliminate coliform contamination by processing in the container; whilst the total number of organisms present was rather higher than in H.T.S.T. process cream, the particular thermoduric types did not proliferate rapidly enough to produce marked deterioration of keeping quality. These storage tests at atmospheric temperature emphasized the practical importance of eliminating the flora associated with post-heating contamination.

The data in Table 5 have also been presented on a monthly basis and show the very pronounced effect of high atmospheric temperatures during the summer months. The can samples from the H.T.S.T. plant showed marked deterioration at atmospheric temperatures from May to September, whilst samples direct from the H.T.S.T. plant discharge or holder

processed in a sealed container probably showed striking changes during a somewhat narrower period in June, July and August.

It was significant, however, that even the most satisfactory original product might still

Table 5. *Seasonal variations in quality of cream after storage for 24 hr. at atmospheric temperature*

(1) H.T.S.T. process, double-heat treatment

Month	Direct-plant samples: No. of samples	Mean colony count	No. of coliform positives	Grading test: 1	2	3	4
Jan.	8	170	.	7	.	.	1
Feb.	10	320	.	8	1	.	1
March	14	450	.	14	.	.	.
April	10	360	.	10	.	.	.
May	13	130	.	10	2	1	.
June	38	4,470	3	24	8	3	3
July	42	870	.	39	1	2	.
Aug.	7	3,800	.	5	2	.	.
Sept.	20	2,570	1	14	4	2	.
Oct.	6	440	.	4	1	1	.
Nov.	6	60	2	4	.	1	1
Dec.	6	260	.	4	2	.	.
All samples	180	890	6	143	21	10	6
			3·4%				8·9%
	Cans filled direct from plant						
Jan.	13	210	2	7	2	2	2
Feb.	10	970	1	9	.	.	1
March	9	1,200	.	8	1	.	.
April	10	1,130	.	5	4	.	1
May	26	23,990	13	9	3	4	10
June	27	21,880	11	5	4	8	10
July	27	58,880	5	1	5	13	8
Aug.	17	74,130	5	1	2	10	4
Sept.	22	6,460	.	1	5	15	1
Oct.	11	440	2	6	1	3	1
Nov.	10	190	.	9	.	.	1
Dec.	12	490	1	10	.	1	1
All samples	194	3,550	40	71	27	56	40
			20·6%				49·5%

(2) Holder process. Samples processed in sealed container

Month	Direct-plant samples: No. of samples	Mean colony count	No. of coliform positives	Grading test: 1	2	3	4
Jan.	6	710	1	5	1	.	.
Feb.	6	3,680	.	6	.	.	.
March	4	600	.	4	.	.	.
April	8	2,200	.	8	.	.	.
May	6	1,500	.	5	1	.	.
June	11	5,300	.	9	1	1	.
July	9	36,300	1	5	2	.	2
Aug.	7	162,000	.	2	2	1	2
Sept.	4	35,900	.	2	2	.	.
Oct.	4	1,430	.	4	.	.	.
Nov.	3	5,400	.	3	.	.	.
Dec.	4	10,960	.	4	.	.	.
All samples	72	5,990	2	57	9	2	4
			2·8%				8·3%

possess a doubtful margin of safety during very hot weather, whilst distribution should present no difficulty during the winter months. The figures suggested that August was the most difficult month—a fact which has long been well known to trade operators. In this connexion it must be remembered that cream may be exposed to atmospheric temperature during distribution for longer periods than is heat-treated milk.

To summarize, bacteriological contamination which has the greatest effect upon keeping quality is largely derived from a number of successive sources following the heat-treatment process; by close bacteriological control of these handling processes, combined with grading tests of individual batches, such contamination may be reduced to very small dimensions and may only be detected with certainty by ageing of samples before testing, whilst complete elimination can only be ensured by heating and cooling the cream in the sealed distributive container. Nevertheless, during hot summer weather (when demand for cream is highest) proliferation of bacteria during distribution may be so rapid that the most stringent bacteriological precautions may be insufficient in themselves to ensure a desirable margin of keeping quality. Most cream distributors have probably reached similar conclusions on the basis of commercial experience.

Consequently it is surprising that in Britain so little serious attention has been given to the possibilities of refrigerated transport as an aid to cream distribution. If cream processed under good bacteriological conditions could be maintained subsequently at a reasonably low temperature during distribution, adequate keeping quality could be guaranteed at all times and popular demand could no doubt be diverted from the inferior tinned sterilized product. This problem has been solved by ice-cream distributors and suitable adaptations to the requirements of the cream trade might well repay early development. In the past such developments have doubtless been hindered by the predominance of rail transport at some stage of the distribution chain.

SUMMARY

1. The special conditions of the cream trade are discussed. Keeping quality is a major consideration which influences all operations and it has been shown that unusually high bacteriological standards of handling are essential.

2. The requirements for bacteriological control testing are indicated, together with the limitations of existing standard methods.

3. A rough sorting test has been developed which permits sufficiently rapid estimation of the probable keeping quality of heat-treated cream and which is applicable to routine grading of numbers of individual units.

4. Data from 2558 samples of heat-treated cream are presented to show the relationship between the colony count, coliform test, grading test, and keeping quality.

5. Possible sources of bacteriological contamination have been indicated by the examination of 2337 plant samples. Commercial processing methods are discussed and satisfactory practical methods of operation have been developed to ensure a minimal bacterial population.

6. The keeping quality of cream has also been studied in relation to the effects of cold storage before distribution and of exposure to atmospheric temperature during distribution.

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365. THE BACTERIOLOGICAL QUALITY OF BRITISH SPRAY-DRIED MILK POWDER

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The increased use of dried-milk products in human feeding during recent years stimulated investigations into and more rigid control of the bacteriological quality.

Spray-dried milk powder from the same eight British plants has been examined for bacteriological quality at this Institute in several different years and the results show the marked improvements which have been made.

In a co-operative experiment undertaken in 1942 at a Kestner plant (1) the preheating of the milk to 190° F. was shown to result in a powder of much lower count than when the preheating temperature was 165° F. In 1943, a similar experiment at a Krause plant (2) using five preheating temperatures, i.e. 200, 190, 180, 170 and 160° F., showed clearly that the preheating temperature has a definite influence on the numbers of organisms surviving in the milk powder and that with a very poor quality raw milk, a preheating temperature greater than 180° F. was necessary to produce a dried milk of low bacterial count.

The use of a preheating temperature of 190° F. was introduced at all spray-drying plants in 1943 and Crossley (3) has shown that the improvements in the bacteriological quality of the milk powder from the Kestner plant resulting from the use of the higher preheating temperature associated with careful control in the cleaning and sterilization of the plant, have been satisfactorily maintained over a period of 2 years.

It may, then, be of interest to record the beneficial effect of the introduction of high-temperature preheating on milk powders from other spray-drying plants, and to compare them with milk powders from the same plants examined in previous years.

Experimental

Sampling

Weekly samples of spray-dried milks were obtained from eight plants in Great Britain between June and September 1942 and April to November 1943, i.e. immediately before and during the first year of the introduction of the preheating temperature of 190° F.

In 1942 three samples were obtained at intervals during a single day's run and in 1943 single samples from about the middle of a run were examined.

Methods of examination

All dried milks were reconstituted at laboratory temperature by a method similar to but differing in detail from that used by Nichols (4) in that 10 g. powder were reconstituted in 90 ml. quarter-strength Ringer solution and the degree of shaking (without beads) was standardized at 50 times in 12–15 sec. with an excursion of 1 ft.

The count was determined on Yeastrel milk agar plates incubated for 3 days at 37° C. and 2 days (1942) or 3 days (1943) at 55° C. Later results have shown (5, 6) that the effect of preheating temperature on the bacterial population may be more clearly demonstrated

when plates are incubated at 30° C. but no comparative figures before 1943 are available at this temperature.

Results

The mean and standard deviations of the plate counts for all spray-dried milk powders examined in 1942 and 1943 are given in Table 1 in which the figures obtained by Nichols(4) in 1937–8 are included for comparison.

Table 1. *Plate count per g. spray-dried milks from all plants*

Year	No. of samples	Mean log count	S.D.	Geometric mean	S.D. range*	% full-cream milk powders
At 37° C.						
1937–8	405†	5·65	0·82	446,700	67,600–2,950,000	10
1942	216	4·42	1·03	26,300	2,460–281,800	46
1943	144	3·59	0·73	3,800	710–20,420	100
At 55° C.						
1937–8	254†	3·94	0·84	8,710	1,260–60,260	10
1942	212	3·01	1·21	1,020	60–16,600	45
1943	136	2·66	0·49	460	150–1,400	100

* Mean count ± standard deviation, which indicates the range within which two-thirds of the counts should normally fall.

† Data calculated from paper by Nichols(4).

It is evident from this table that a marked improvement in bacteriological quality had been achieved between 1937–8 and 1942 although the standard deviation was still high, an indication that the extent of the improvement varied at different plants. An analysis of the results for the eight individual plants (Table 2) confirms this fact. Some plants in 1942 were still producing spray-dried milk powder with a mean plate count of several hundred thousand bacteria per g. while a few were producing powder containing only a few thousands per g.

Table 2. *Plate count per g. spray-dried milks from individual plants*

Plant	Year	No. of samples	Mean log count	S.D.	Geometric mean	S.D. range
A*	1937–8	24	6·05	0·71	1,112,000	218,800–5,129,000
	1942	34	4·49	0·94	30,900	3,550–269,200
	1943	19	3·59	0·67	3,890	820–18,200
B	1937–8	63	5·74	0·73	549,500	102,300–2,951,000
	1942	41	4·72	0·78	52,480	8,710–316,200
	1943	19	3·90	0·47	7,940	2,690–23,440
C	1937–8	12	4·89	1·95	77,600	870–6,918,000
	1942	20	5·30	0·48	199,500	66,070–602,600
	1943	12	3·34	0·32	2,190	1,050–4,570
D	1937–8	138	5·93	0·91	851,000	104,700–6,918,000
	1942	18	5·11	1·24	128,000	7,410–26,300
	1943	19	3·80	0·97	6,310	3,240–9,330
E	1937–8	66	4·07	0·39	11,750	4,790–28,840
	1942	20	4·48	0·39	30,200	12,300–74,130
	1943	21	3·63	0·50	4,270	1,260–12,590
F	1937–8	90	6·19	1·32	1,549,000	74,130–32,360,000
	1942	28	3·83	0·59	6,670	1,740–26,300
	1943	16	3·74	0·23	5,550	3,240–9,330
G	1942	19	4·94	0·66	87,100	19,050–398,100
	1943	15	4·31	0·21	20,420	12,590–33,100
H	1937–8	12	4·77	0·61	58,880	14,450–239,900
	1942	36	3·29	0·98	2,000	210–19,050
	1943	23	2·67	0·59	470	120–1,820

* The letter indicating individual plants does not necessarily coincide with that used by Nichols(4) in her report on the 1937–8 results.

After the introduction of the high preheating temperature (190° F.) in 1943 there was a further definite decrease in the numbers of organisms surviving in the powders and a rather lower standard deviation, indicating a more uniform product from the bacteriological standpoint. The powder examined in 1943 was, in addition, more uniform in that it was all prepared from full-cream milk, whereas in 1942, 52% and in 1937–8 about 90% of the samples examined were separated milk powders. As Nichols (4) has shown by direct comparison and as more recent general experience has confirmed, the plate count of separated milk powder is usually higher than that of full-cream milk powder. This would then in part account for the fall in surviving bacteria, but figures given in Table 2 for individual plants show the marked improvement in plate count at 37° C. between 1937–8 and 1942 at plants D, E and F which were drying separated milk in both periods and between 1942 and 1943 at plants A and B which were drying full-cream milk in each of these years. It is thus evident that the improvement in bacteriological quality has been due mainly to the greater care taken in plant management associated with the high preheating temperature.

Only 39 samples of milk powder from three of the plants were examined in 1944. The mean count at 37° C. for these samples (including 14 from separated milk) was 3630, with a standard deviation range of 930–14,130. These figures are in agreement with those obtained in 1943 and show that the low bacterial count of the powder was being maintained at three at least of the plants.

The author wishes to express her thanks to the manufacturers for their co-operation in supplying the samples of spray-dried milks.

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366. DYE-REDUCTION TESTS IN THE BACTERIOLOGICAL EXAMINATION OF DRIED MILK

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Nichols, in her bacteriological studies of spray-dried milks (1), determined the methylene-blue reduction time of the freshly reconstituted milks at 37 and 55° C. The weighted mean reduction time at 37° C. was 8·1 hr. (range 3–14 hr.) for spray-dried milks having a mean log count equivalent to 800,000/g. A reasonably good agreement (correlation coefficient $r = -0{\cdot}72$) was obtained between the reduction time and the log plate count at 37° C.

At 55° C. the mean reduction time was much longer, over 30 % of the samples requiring at least 12 hr. and none less than 4½ hr. and the correlation with the plate count was poor ($r = -0{\cdot}22$).

The spray-dried milks now being produced have, however, a very much lower bacterial content (2) and a direct determination of the methylene-blue reduction time of the freshly reconstituted milk would obviously take too long to be of practical value as a routine test. On the other hand, the accurate reading of plates prepared from low-count milk powder is frequently difficult owing to the opacity of the medium (often containing 1 ml. of reconstituted milk) and the relatively frequent occurrence of spore-formers increases these difficulties. Reconstitution at 50° C. with a short holding time at that temperature (3, 4) effects some improvement in the reading of the plate count but does not entirely overcome the difficulties.

It was, therefore, decided to investigate the possibility of applying the methylene-blue reduction test to reconstituted milks after ageing to allow development of the bacterial flora and so obtain a shorter reduction time which might be of practical value in assessing the bacteriological quality of reconstituted milks.

Milks prepared from various types of milk powder have been examined after ageing at different temperatures and the plate count and keeping quality determined to serve as standards of reference in assessing the value of the reduction test.

Experimental

Samples

A small number of samples of spray-dried full-cream and separated milks from four plants was examined within 1 week of production and a larger number of spray-dried milks from eight plants examined 6 months after production. Roller-dried full-cream milks (National Dried Milk) were examined during the period of their availability for infant feeding, i.e. within 6 months of production.

Methods

Plate counts on the freshly reconstituted milks were determined on Yeastrel milk agar after incubation for 3 days at 37° C., 5 days at 30° C. and 3 days at 55° C. Portions of the same reconstituted milk were then transferred to (*a*) bottles, to be incubated at 15·5° C. for the determination of time to clot on boiling, (*b*) dye-reduction tubes, for incubation

with methylene blue and with resazurin at 15·5° C. (keeping quality), and (*c*) dye-reduction tubes, for incubation overnight (18–20 hr.) at 55, 37, 30, 22 and 15·5° C.

For (*c*) two tubes were incubated at each temperature and next day 1 ml. methylene-blue solution was added to one and 1 ml. resazurin solution to the second and the reduction test at 37° C. immediately carried out in the usual way. The dye-reduction tests on reconstituted milk aged at 55° C. were later done at 55° C. instead of at 37° C. The strength of the methylene-blue and resazurin solutions were those prescribed for raw milk by the Ministry of Health (5) and in the National Milk Testing Scheme (England and Wales) (6) respectively.

As this investigation was made at the same time as that on technique (4), duplicate tests were done on the milks reconstituted at room temperature (technique A) and those reconstituted at 50° C. (technique B).

Readings of the dye-reduction tests were taken immediately and every 5 min. to 30 min., at 45 and 60 min. and thereafter at half-hourly intervals to 6 hr. Tubes were inverted at every ½ hr. Indeterminate results, i.e. reduction times over 6 hr. have not been included in the statistical calculations.

Keeping-quality determinations were made twice a day, i.e. at 9.30 a.m. and 4.30 p.m. The time interval between each reading was therefore alternately 17 and 7 hr. In the statistical calculation these readings have been regarded as being at equal intervals of 12 hr. although rather more accurate correlations would have been obtained if the intervals had been regarded as ⅔ and ⅓ days.

RESULTS

Effect of ageing on dye-reduction times

At 37° C. Incubation at 37° C. for 18–20 hr. proved too severe as 20% of the roller-dried, over 90% of the spray-dried full-cream and 50–60% of the spray-dried separated milks clotted either during incubation or during the subsequent dye-reduction test.

With roller-dried milks between 70 and 90% (varying with the technique of reconstitution) of 101 samples reduced methylene blue within 15 min. although the log plate count of the freshly constituted milks varied from 1·50 to 4·50 and the correlation coefficient was therefore very low ($r = 0{\cdot}16$, $0{\cdot}18$). Similar results were obtained using resazurin, with a rather higher correlation coefficient ($r = -0{\cdot}42$ to $-0{\cdot}47$) due to the lower incidence of delayed reduction times. Occasionally with resazurin and relatively frequently with methylene blue a long reduction time was recorded due to rapid partial decolorization followed by delay in the removal of the last trace of colour. This was often but not invariably associated with the clotting of the milk.

At 30° C. Though rather less severe than incubation at 37° C., 40–50% of the spray-dried full-cream milks incubated at 30° C. for 18–20 hr. clotted either during ageing or testing but few of the roller-dried milks actually clotted. Delayed reduction times were again recorded although a high proportion—84% of roller-dried milks, 70% of spray-dried full-cream and 60% of spray-dried separated milks—reduced methylene blue within 15 min. Roller-dried milks showed a moderate correlation ($r = 0{\cdot}40$–$0{\cdot}50$) between plate count at 30° C. and methylene-blue reduction time determined at 37° C. after ageing at 30° C. The degree of correlation of reduction time was similar ($r = -0{\cdot}51$) with plate counts made at 30 and at 37° C. Results with resazurin were similar.

At 22° C. Clotting during examination was eliminated by incubation at 22° C. On the

other hand, a few samples, i.e. about 6% of the roller-dried and 8% of the spray-dried milks had reduction times greater than 6 hr.

Table 1 summarizes the results obtained under these conditions. The reduction time showed considerable variation with a mean time for spray-dried milks of 2½–3 hr. and for roller-dried milks of 3–3½ hr. Such reduction times are within a range which would be practicable for the routine testing of dried milks, but they showed a very poor correlation (Table 1) with the plate count at 30 or 37° C. and with the keeping quality whether determined by dye-reduction test or by clot on boiling. This lack of correlation was obtained with all types of dried milks and was not appreciably affected by the method of reconstitution.

Table 1. *Correlation between* M.B.R.T. *after ageing at* 22° C. *and plate count or keeping quality*

Correlation of	No. of samples		Correlation coefficient (r)		Mean log plate count/g. or mean K.Q. in hr.		Standard deviation		Mean M.B.R.T.		Standard deviation	
	A*	B*	A	B	A	B	A	B	A	B	A	B
Spray-dried milks:												
M.B.R.T. with plate count 37° C.	87	85	−0·27	−0·19	4·68	4·04	2·55	2·91	2·88	2·65	2·98	2·81
M.B.R.T. with plate count 30° C.	83	81	−0·20	−0·17	4·77	4·33	2·40	2·47	2·99	2·79	3·16	2·92
M.B.R.T. with keeping quality (M.B.)	91	90	0·52	0·30	68	68	2·27	1·81	2·90	2·74	2·93	2·86
M.B.R.T. with keeping quality (C.O.B.)	91	90	0·12	0·33	75	76	1·10	1·32	2·90	2·78	2·93	2·86
Roller-dried milks:												
M.B.R.T. with plate count 37° C.	130	123	−0·42	−0·32	3·37	3·70	2·70	3·00	3·56	3·14	3·06	3·06
M.B.R.T. with plate count 30° C.	131	125	−0·45	−0·28	3·45	3·61	2·30	2·54	3·18	3·13	2·53	2·26
M.B.R.T. with keeping quality (M.B.)	138	136	0·33	0·55	65	68	1·06	1·21	3·21	3·13	2·94	3·17
M.B.R.T. with keeping quality (C.O.B.)	135	136	0·11	0·23	78	78	1·10	1·10	3·16	3·10	2·50	2·06

* These letters denote that samples for test were reconstituted at laboratory temperature (technique A) and at 50° C. (technique B).

At 15·5° C. After incubation for 20 hr. at 15·5° C., 55–65% of all spray-dried milks and 44% of the roller-dried milks failed to reduce methylene blue at 37° C. within 6 hr. Moreover, the plate count of those samples not reducing methylene blue within 6 hr. varied over a wide range and there was obviously no correlation between the count and the reduction time.

On the other hand, since ageing took place at the same temperature as that used for keeping-quality determinations, a reasonably good agreement between reduction time at 37° C. and keeping quality would be expected. For spray-dried milks this agreement was in fact quite satisfactory. Those samples (35–45%) giving a definite reduction time at 37° C. (i.e. within 6 hr.) gave a correlation coefficient with keeping quality as determined by the methylene-blue method of 0·72–0·84 and as determined by clot on boiling of 0·44. For similar roller-dried milks (56%) the correlation with keeping quality (methylene-blue technique) was lower ($r = 0{\cdot}40$–$0{\cdot}50$).

All these samples (spray and roller-dried) had a keeping quality of 60 hr. or less, whereas of those giving a reduction time at 37° C. greater than 6 hr., only 8% spray and 6%

roller-dried milks had a keeping quality of less than 60 hr. There was, however, considerable overlapping at 60 hr., i.e. in the range within which most of the samples (60%) occurred.

At 55° C. The examination by reduction test at 37° C. of reconstituted milks aged at 55° C. gave very unsatisfactory results bearing no relation to the plate count at 55° C. of the freshly reconstituted milk. Delayed reduction was frequently shown, e.g. with resazurin a particular disk number was often held for several hours and most samples reducing resazurin completely in up to 3½ hr. gave a disk reading of ½ before incubation began at 37° C.

An incubation temperature of 55° C. was then used for the dye-reduction test on the aged milk, but delayed reduction times were again obtained. With recently manufactured spray-dried separated milks 50% failed to reduce methylene blue in 6 hr.; with full-cream milks 50% clotted during examination, while with 6 months old powder 70–80% of the aged samples reduced within 1½ hr. but the corresponding log-plate counts varied from 1·76 to 4·25 and there was obviously no correlation between plate count and reduction time.

All roller-dried milks (70 samples) reduced within 2 hr. but the reduction times bore no relation to plate count and delayed end-points were of frequent occurrence.

Nine roller-dried and ten spray-dried full-cream milks were examined by direct dye reduction test at 55° C. on the freshly reconstituted milks. No reduction occurred within 6 hr. but all samples had decolorized by the next day, i.e. in 18 hr. even when the plate count at 55° C. was less than 100/g.

DISCUSSION AND CONCLUSIONS

An attempt has been made to estimate the bacteriological quality of milk powders using a dye-reduction technique. The ageing of reconstituted milk before testing has been investigated, to determine the conditions most suitable for the use of this technique in the routine bacteriological examination of dried milks.

Since it is both usual and advisable to use dried milk immediately after reconstitution, the plate count at this stage seems the most satisfactory standard by which to assess the value of the methylene-blue test. Tests for keeping quality at 15·5° C. have also been included as indicators of the potential bacteriological changes which might be expected in the reconstituted milk. The results of such tests, however, vary within a relatively narrow range and are not a very sensitive indicator of quality. Indeed Nichols (1) excluded keeping quality from the statistical treatment of her results on account of this lack of differentiation between samples.

A temperature of 22° C. appears to yield an incubated milk giving the most suitable range of dye-reduction times. At higher temperatures, including 55° C., clotting of the milk and delay in the final stages of reduction interfere with the test, while at lower temperatures (15·5° C.) incubation for 18–20 hr. is, in most samples, insufficient to produce a degree of bacterial growth capable of reducing methylene blue within 6 hr.

Unfortunately, the correlation between reduction time after ageing at 22° C. and plate count at either 30 or 37° C. was poor for all types of milk and the results cannot therefore be accepted as giving a true assessment of the bacteriological quality of the milk powder or, more strictly, an assessment bearing any relation to the present accepted plate-count technique. The correlation with keeping quality at 15·5° C. whether determined by dye-reduction test or by clot on boiling was also unsatisfactory.

SUMMARY

1. Over 100 samples of roller-dried full-cream milk and rather less than 100 samples of spray-dried milk reconstituted and incubated for 20 hr. at 55, 37, 39, 22 and 15·5° C. have been examined by the methylene-blue or resazurin-reduction tests at 37° C. The results have been compared with the plate count at 37 or 30° C. of the freshly reconstituted milk and with the keeping quality at 15·5° C.

2. A range of reduction times suitable for routine use was obtained after incubation at 22° C., but the results showed a very poor correlation with plate count at 37 or 30° C. and with keeping quality.

3. After incubation at 55, 37 and 30° C. the reduction times were too short, often showed delayed end-points, and the milk frequently clotted during examination.

4. Results with resazurin were similar to those using methylene blue and no significant differences resulted from the two different methods of reconstitution.

The author desires to express her thanks to the manufacturers for the supplies of the spray-dried milks and to the Ministry of Food for the samples of National Dried Milk.

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367. BACTERIAL GROWTH IN RECONSTITUTED SPRAY-DRIED MILK

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The increased use of dried milk has emphasized the importance not only of the numbers of organisms in milk powder but also of the types which grow most readily after reconstitution.

An important advantage of milk powder as compared with raw milk is that, from a bacteriological point of view, it keeps indefinitely while in the dry state. It is frequently not sufficiently realized that for many purposes it is not necessary to reconstitute the milk powder before use and further, that once reconstituted the milk will no longer keep indefinitely.

The possibility of reconstituted milk being held for some time before use and the implication of milk-containing foods in food-poisoning of the enterotoxin type (particularly in America) make it important that the types of surviving organisms which would grow readily after reconstitution should be known.

The present report deals with such an investigation using British spray-dried milks manufactured in 1942. Both quantitative and qualitative bacteriological examinations were made of reconstituted milks, freshly prepared and after ageing at 15·5 and 22° C. to simulate storage under cool and rather high household temperatures.

No attempt was made to determine whether the organisms isolated from the reconstituted milks were in fact capable of producing an enterotoxin.

Experimental methods

Sampling

Weekly samples of dried milks were obtained from eight spray-drying plants in Great Britain between June and September 1942. These samples were taken by plant personnel directly from the drier and were therefore subject to the minimum degree of handling. About 46 % of the powders were made from full cream and the remainder from separated milk.

Methods of examination

10 g. powder were reconstituted in 90 ml. quarter-strength Ringer solution at laboratory temperature by shaking 50 times in 12–15 sec. with an excursion of 1 ft.*

The total count was determined in Yeastrel milk agar plates incubated for 3 days at 37° C. or 2 days at 55° C. A small number of duplicate total counts was also made in blood agar and any β-haemolytic colonies noted. The spore count on blood agar was determined after heating the milk at 80° C. for 15 min. Yeast and mould counts were obtained on beer-wort agar (pH 3·5) after 5 days' incubation at 22° C. The presumptive coliform test was made on 1 ml. reconstituted milk.

The plate count at 37° C. on Yeastrel milk agar was determined for a number of samples of the milk after ageing for 24 hr. at 15·5 and 22° C. The keeping quality at 15·5° C. was

* This work was done before the introduction of reconstitution at 50° C. as the standard procedure.

determined as the time taken before the milk clotted on boiling. No tests for palatability were made.

For a random selection of the samples, the majority flora immediately after reconstitution and again after ageing at 15·5, 22 and 37° C. was determined by streaking milk agar plates with a standard loopful of the milk. Colonies of the predominating types of organisms were picked off into litmus milk and examined. This method was chosen as being less laborious than picking colonies from the counting plate since it gave fewer colonies and these of the predominating types. In practice it was found that occasionally very few colonies grew from the inoculum of the freshly prepared milk while spore-forming bacilli were apt to overgrow other compact types in inocula from aged milk. Duplicate examinations were done in a few instances from the counting plate and these agreed with the results obtained on the streak plate.

Results

Quantitative examination

(1) *Freshly reconstituted milks*

The mean plate counts of the freshly reconstituted milks expressed per ml. (i.e. one-tenth of that per g. powder) are given in Table 1. The plate counts of these powders were considerably higher than from the same plants in subsequent years (1) and the samples may therefore be taken as representing a poorer quality than could be obtained from British plants at the present time.

Table 1. *Plate count per ml. of reconstituted milks from spray-dried powder*

Type of count	No. of samples	Mean log count	S.D.	Geometric mean	S.D. range
Total (milk agar 3 days 37° C.)	216	3·42	1·03	2,630	250–28,180
Total (milk agar 2 days 55° C.)	212	2·01	1·21	100	10–1,660
Spore count (37° C.)	152	1·54	0·80	35	10–220
β-haemolytic spore-formers (blood agar 37° C.)	115	0·17	0·96	1·5	0–14

The total count per ml. for 92 of the samples which were examined on both milk agar and blood agar at 37° C. gave a mean log count of 3·54 and 3·50 respectively. The standard deviation (S.D.) range (within which two-thirds of the counts would normally fall) expressed in terms of actual count was 250–40,740 on milk agar and 350–34,670/ml. on blood agar. It is evident that blood agar offers no advantage over milk agar in the determination of total count. β-haemolytic colonies were present in 30, i.e. one-third, of these samples but all these colonies were typical of spore-forming bacilli. β-haemolytic streptococci or staphylococci were therefore not present in the reconstituted milk.

Although the number of samples was not exactly comparable, the mean spore count at 37° C. (Table 1) may be taken as about one-seventieth of the mean total count. Of these approximately 5% produced β-haemolysis on blood agar.

Very few moulds were present in these milks. Of 119 samples examined 36% had no moulds, 61% had 10 or under and only 3% gave more than 10 mould colonies per ml. of reconstituted milk. One sample gave 10 yeast colonies per ml. and 1 yeast was present on two other plates. In the remaining milks no yeasts were present in 1 ml.

The presumptive coliform test gave a positive test in 1 ml. in only 10 out of 193 samples examined and these were confined to the powders from three of the eight plants. Of the ten positive tests, four were not examined further, one was a false positive, and the remaining five were due to either *Bact. coli* I or *Bact. aerogenes* I.

The keeping quality at 15·5° C. assessed from the time of reconstitution to clot on boiling was 3–4 days for 83% of the samples, only 7% clotting in less than 3 days.

(2) *Reconstituted milks aged for 24 hr.*

The mean plate counts of 42 samples after 24 hr. at 15·5° C. and at 22° C. compared with that of the same samples immediately after reconstitution are given in Table 2. The counts of the milks when held at 15·5° C. were essentially unchanged, but at 22° C. considerable growth had taken place and the mean count increased over 2000-fold. The plates prepared from a few of these milks after ageing at 22° C. showed no colonies of spore-forming bacilli but of 24 samples in which the incidence of such colonies was noted, half showed over 50% and five over 90% of the total count to be of this type.

Table 2. *Plate count per ml.* (37° C.) *of reconstituted milk, freshly prepared and after ageing for 24 hr. at 15·5 and 22° C.*

State of reconstituted milk	No. of samples	Mean log count	S.D.	Geometric mean	S.D. range
Fresh	42	3·82	0·81	6,610	1,000–42,660
Aged 24 hr. at 15·5° C.	42	3·79	0·93	6,170	720–54,950
Aged 24 hr. at 22° C.	42	7·19	1·09	15,488,000	1,258,900–190,550,000

Six reconstituted milks with counts per ml. ranging from 30 to 84,000 had clotted in 24 hr. when aged at 37° C. Streptococci formed the main flora in such milks.

It is evident therefore that, while very little growth normally occurs at 15·5° C. in 24 hr., at higher temperatures—such as that of a rather warm room—reconstituted milks will rapidly deteriorate.

Qualitative examination

In general the main flora of the freshly reconstituted milk varied with its source, the flora of the individual samples from each plant being generally similar and in some instances definitely characteristic.

In Table 3 the main types of organisms occurring in samples from each plant are given, together with those which occurred in smaller numbers and less regularly.

It will be noted that two organisms were of very frequent and general occurrence, i.e. *Streptococcus faecalis* (or closely related types of enterococci) and a micrococcus showing the characteristics of *Micrococcus casei liquefaciens* Orla-Jensen. The latter occurred as tetrads and as irregular groups of cocci in different strains. These two morphological types were otherwise identical and have been classed together. Both were very actively proteolytic in gelatin and litmus milk.

Other micrococci, some pigmented, large (0·8–1·0μ diam.) and forming tetrads, others non-pigmented, smaller and usually in irregular groups, were encountered less frequently except in milks from plant D. Here the main flora was a large slow-growing tetracoccus, non-saccharolytic, which failed to produce diastase or reduce nitrate to nitrite. Some strains produced a very slight and slow liquefaction of gelatin and all produced a pale pink pigment. This organism, however, survived heating in litmus milk for 30 min. at 62·5° C. The slow rate of growth may account for its absence among the colonies isolated after ageing at 22° C.

Of streptococci other than *Streptococcus faecalis* small numbers of less well-defined types (possibly of the *lactis* group) were isolated and *Str. thermophilus* occurred in a few samples usually associated with a high total count.

As would be expected from the plate counts, the main types of organisms present after ageing for 24 hr. at 15·5° C. were identical with those in the freshly reconstituted milk with, in addition, a small number of spore-forming bacilli not isolated from the fresh milk.

After 24 hr. at 22° C. the same main types were usually present but were not necessarily the most numerous owing to the marked increase in numbers and variety of spore-forming bacilli. Most of these spore-forming bacilli fell into one main group, possibly the unidentified *Y* group of Gibson's classification (2), though a few might be considered as of the *B. megatherium* group. All these produced acid in glucose broth and were Voges-Proskauer

Table 3. *The predominating types of organisms in reconstituted spray-dried milk when fresh and after ageing for* 24 *hr. at* 15·5 *and* 25° C.

Plant	Reconstituted milk (fresh)	After 24 hr. at 15·5° C.	After 24 hr. at 22° C.
A	*Str. faecalis.* Also *B. licheniformis* group; streptococcus of 'lactis' group; yellow micrococcus (tetrads)	*Str. faecalis.* Also strep. of 'lactis' group; *Micrococcus casei liquefaciens* (Orla-Jensen); spore-formers of type II and *B. licheniformis* group	*Str. faecalis*; *Micrococcus casei liq.* O.-J.; spore-forming bacilli types II, V, VII and occasionally *B. licheniformis* and *B. pumilus* groups
B	*Str. faecalis.* Also *B. licheniformis* group; yellow micrococcus (tetrads); *Achromobacter* sp.	As fresh. Spore-forming bacilli of *B. licheniformis* group and types V and occasionally I, also *Micrococcus casei liq.* O.-J.	*Str. faecalis*; strep. of 'lactis' group; spore-forming bacilli of *B. licheniformis* group; type V and occasionally type II
C	*Str. thermophilus.* Also *Str. bovis* and enterococci; pale pink micrococcus (tetrads); *B. licheniformis* group and occasionally type I	As fresh	*Str. faecalis*; spore-forming bacilli of type I and *B. licheniformis* group; other streps.
D	Pale pink micrococcus (tetrads); *Str. thermophilus*; *Micrococcus casei liq.* O.-J.	As fresh. More frequently *Micrococcus casei liq.* O.-J.; spore-forming bacilli of types II, III, V and VI	*Str. faecalis*; spore-forming bacilli of types I, II, III and VI. Also *Micrococcus casei liq.* O.-J.
E	*Str. faecalis.* Also strep. of 'viridans' group; pale pink micrococcus	Spore-forming bacilli of types II, III, V and VII. Also *Str. faecalis*; *Micrococcus casei liq.* O.-J.	Spore-forming bacilli; types I, II, III, V, VII and *B. fusiformis* group. Also *Str. faecalis*; *Micrococcus casei liq.* O.-J.
F	*Micrococcus casei liq.* O.-J. Also yellow micrococcus (tetrads); *B. fusiformis* group	*Micrococcus casei liq.* O.-J. Occasionally other micrococci and few streps.	As 15·5° C. and spore-forming bacilli; types I, IV, VII and *B. fusiformis* group
G	*Str. faecalis*; *Micrococcus casei liq.* O.-J. Also pale pink micrococcus; *Str. thermophilus*	*Str. faecalis*; *Micrococcus casei liq.* O.-J.; spore-forming bacilli; types I, II, III and V	*Micrococcus casei liq.* O.-J.; *Str. faecalis* spore-forming bacilli; types I, II, III and VII
H	*Micrococcus casei liq.* O.-J.; *Str. faecalis.* Also *B. pumilus*; *Str. thermophilus*	*Micrococcus casei liq.* O.-J. Also *Str. faecalis*; spore-forming bacilli; type I and *B. cereus* group	*Micrococcus casei liq.* O.-J.; spore-forming bacilli; types I, II, III and of *B. pumilus* and *B. fusiformis* groups

negative; rods from glucose agar stained uniformly with fuchsin. They produced β-haemolysis on blood agar, clotted litmus milk (with or without acid production) with subsequent digestion of the clot, and liquefied gelatin.

This main group could be subdivided on the basis of lactose fermentation, diastase production and reduction of nitrate to nitrite while within each subgroup sucrose fermentation was variable. The subgroups were as follows:

I. No acid in lactose, no diastase, nitrate reduced to nitrite.

II. As I but diastase produced.

III. No acid in lactose, diastase produced, no nitrite from nitrate.

IV. As I but no nitrite from nitrate.

V. Acid in lactose, diastase produced, nitrate reduced to nitrite.

VI. As V but no diastase.

VII. As V but no nitrite from nitrate.

Strains falling into subgroups IV and VII were few, most being approximately equally distributed among the other subgroups.

Only occasional strains of the *B. pumilus*, *B. cereus* and *B. fusiformis* groups were encountered but strains apparently of the *B. licheniformis* group were the main type of spore-forming bacillus in the milk from two plants. Indeed, in freshly reconstituted milks of low count from these two plants examined at a later date, they were present in almost pure culture. The strains included in the *B. licheniformis* group grew typically as rather yellowish or yellowish brown small non-spreading colonies of dull, wrinkled appearance, adherent to the medium and very difficult to emulsify. They produced acid in glucose, usually produced diastase, reduced nitrate to nitrite and liquefied gelatin very slowly if at all. They were usually without effect on blood agar though some strains showed slight haemolysis after 48 hr. Many strains grew readily at 55° C.

DISCUSSION AND CONCLUSIONS

In Britain reports of outbreaks of food-poisoning of the gastro-enterotoxic type are much less common than in America but available evidence suggests that small outbreaks may occur frequently but are not notifiable and therefore do not come to the notice of the health authorities (3).

Stone (4) in his review of 'toxin' outbreaks attributed to raw milk and dairy products in America makes only one reference to dried milk as a possible vehicle but the evidence is entirely presumptive and therefore inconclusive. No other reference in the literature to dried milk in this connexion has been traced but one verbal report has been received in which dried milk in Britain was implicated.

The organism most frequently responsible for outbreaks of enterotoxic poisoning, *Staphylococcus aureus* (or related staphylococci), was not encountered in the present work nor in the earlier work of Nichols (5) and Crossley & Johnson (6) on spray-dried milks.

The main types of streptococci, indeed the predominating flora, in the spray-dried milks of very high count examined by Nichols in 1937–8 were *Str. thermophilus* and *Str. durans*. Later Crossley found the former species associated mainly with very high-count powder and *Str. durans* most common in the rather lower-count milks which formed the bulk of his samples. Streptococci of the 'viridans' and 'enterococcus' groups were less common. Micrococci were regularly encountered and Achromobacter species and aerobic spore-forming bacilli present in small numbers.

In the present work on dried milks from the same plants as in Nichols's survey the mean bacterial count was lower than in Crossley's work. *Str. thermophilus* was uncommon except in a few rather high-count powders while streptococci of the 'enterococcus' group, usually *Str. faecalis* predominated in many of the powders, associated with small numbers of micrococci. In others micrococci (of a relatively few species) were the main types of organisms accompanied by fewer streptococci, other micrococci and spore-forming bacilli.

In the reconstituted milk aged at 22° C. *Str. faecalis*, micrococci and spore-forming bacilli were the predominating organisms. The increase in numbers of the spore-formers

was very marked. Of these organisms, micrococci (other than staphylococci) do not appear to have been implicated in any 'toxin' food-poisoning outbreaks. *Str. faecalis*, however, has been isolated in large numbers from cheese (7, 8) and from evaporated milk (9) which were held responsible for 'toxin' outbreaks but no direct evidence of the toxicity of these strains has been presented.

Spore-forming bacilli have been reported as possible toxic agents. In the report of a discussion at the Royal Sanitary Institute (10) W. M. Scott is quoted as stating that between 1929 and 1937 of 142 'toxin' outbreaks in England, five were associated only with large numbers of spore-forming bacilli of the *B. subtilis* and *B. mycoides* groups. These, in default of other evidence, were provisionally accepted as the causal agent.

Although streptococci and spore-forming bacilli such as occur in dried milk have been found in very large numbers in food responsible for outbreaks of gastro-enterotoxic poisoning, the absence of a really reliable test for enterotoxin has prevented any definite proof of these organisms as the causal agents. Further, even if the kitten test for enterotoxin be accepted, it is not convenient to test the potentialities of large numbers of organisms by this method and no attempt has therefore been made in relation to the particular strains isolated in the present work.

It is obviously undesirable to have large numbers of organisms, whatever their enterotoxic potentialities, present in any foodstuff. The numbers of bacteria in freshly reconstituted milk are lower than those of average quality raw milk. Much of the value of milk powder lies in the ease with which it can be reconstituted as required. It should not be necessary to hold the milk after reconstitution for any considerable period but if this is done it is essential that low-storage temperatures be maintained.

Summary

1. Over 200 samples of spray-dried milks from eight British plants have been examined for total and spore counts, numbers of β-haemolytic colonies and for moulds, yeasts and coliform bacteria.

2. The mean count and standard deviation range for total and spore count are reported. No β-haemolytic colonies other than those due to spore-forming bacilli were detected.

3. After ageing the reconstituted milk for 24 hr. at 15·5° C. the mean count (37° C.) was unchanged; at 22° C. the increase was 2000-fold and at 37° C. the milk had clotted within this time.

4. The predominating organisms in a random selection of the reconstituted milks are described, both for the fresh milk and after ageing at 15·5 and 22° C. A marked increase in spore-forming bacilli was noted at 22° C.

5. The relation of the flora of reconstituted milk to its food-poisoning potentialities is briefly discussed.

The author wishes to express her thanks to Dr A. T. R. Mattick, of the National Institute for Research in Dairying, with whom the above investigation was planned. She also wishes to thank the owners of the plants from which the samples of dried milks were obtained.

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368. DETERIORATION ON STORAGE OF DRIED SKIM MILK

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PART I. INTRODUCTION

BY KATHLEEN M. HENRY, S. K. KON, C. H. LEA AND J. C. D. WHITE

Henry, Kon & Rowland (1, 2) found, by the method of Mitchell (3, 4), that the biological value of the proteins of a bulk of dried skim milk stored at room temperature under conditions which did not exclude atmospheric moisture gradually deteriorated from 88·5 when the powder was a year and a half old to 71·1 three years later. The original moisture content of this powder and the rate of increase was not known, but at the end of the storage period it was 7%.

Findlay, Smith & Lea (5) had already observed that, with whole-milk powder, there was a critical moisture content below which fat deterioration was the first noticeable effect of storage, above which, however, severe non-fatty deterioration was the first to occur on keeping. These changes occurred very slowly at room temperature but much more rapidly at 37 and 47° C. The non-fatty deterioration resulted in unpleasant 'cardboard' or 'gluey' flavours, marked darkening of the powder and a great decrease in the solubility of the protein.

It was realized that the two observations were probably closely related, and a large-scale experiment was arranged by the three Research Institutes concerned to inquire fully into the deterioration on storage of skim-milk powder.

For this purpose a quantity of dried skim milk was prepared and samples of low, medium and high moisture content packed in air and in nitrogen were stored at three temperatures as described in Part II. At frequent intervals sample cans were removed for examination by taste and by various physical and chemical methods (Part III). On the basis of these tests a smaller number of the stored powders was selected for further examination for the nutritive value of the proteins by biological methods (Part IV), and for microbiological assay of 'essential' amino-acids (Part V). A brief discussion of the results obtained by the various methods is given in Part VI. A preliminary account of this work has already been published (6).

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PART II. PREPARATION, PACKING AND STORAGE OF THE EXPERIMENTAL POWDERS

By C. H. LEA and J. C. D. WHITE

(With 1 Figure)

Preparation (J. C. D. White)

For the purpose of the experiment, it was necessary to prepare three batches of spray-dried separated milk powder of at least 1 cwt. each and with moisture contents of the order of 2, 5 and 7%. A Gray-Jensen plant was used.

Bulk whole milk (late April 1945) was separated and dried in three 300 gal. batches. It was decided to use a pre-heating temperature of 165° F. (74° C.) with as short a holding time as possible. (For details of the pre-heating system in this type of drier see Hunziker (1).) In the present experiment, the first 300 gal. of separated milk were circulated from the storage tank through a tubular heater and back to the storage tank until the desired temperature of 165° F. was attained. This operation took about 20 min. 200 gal. were then pumped to the 'concentrator' or 'liquid collector', and thence to the drying-chamber. In the meantime, the remaining 100 gal., which subsequently passed to the 'concentrator' within about 15–20 min., were held at about 160° F. The batch was dried in about 45–60 min. Thus the milk which was dried towards the end of the 'run' was heated for a longer period than that dried at the beginning, but the average duration of the 165° F. pre-heating was about 30 min. The other two batches were treated in a similar way.

It was thought that powders of the requisite moisture contents could be obtained by making some very small alterations in the drying process, but when the drying of the powder was begun, it was found that the lowest moisture content conveniently obtainable was about 2·8% and the highest just under 5%.

One batch of milk was dried to give a powder of the 'low' moisture content, about 180 lb. being collected. The powder was well mixed and packed into cans holding 21 lb. The other two batches of separated milk were then dried to give powders with about 4·7% moisture. These two powders were mixed together very thoroughly in a stainless steel cheese vat to get a homogeneous powder with a uniform moisture content. Half of the mixture was packed into 21 lb. cans and the moisture content of the other half was raised from 4·7 to about 7–8% in the following way.

About 190 lb. of the powder were spread out on two curd-cooling troughs made of tinned steel and having a depth of 9 in. and a combined surface area of about 33 sq.ft. The air of the room was kept humid by water vapour from an open vat of water kept boiling by a steam jet. Every 10–15 min., the powder was thoroughly mixed and built into ridges to increase the surface exposed. On the first day, the powder was exposed for 12 hr., the relative humidity being about 75% for most of the time but rising to 85% towards the end of the period. Representative samples were taken at intervals for the determination of moisture content by heating in an air oven for 3 hr. at 101·5° C. The powder was then filled into cans for the night. Next morning the apparent moisture content of the powder had fallen from 6·2 to 5·8% despite the fact that it had been in tightly closed cans in the interval. This decrease may have been due to crystallization of a small proportion of the anhydrous lactose (cf. below and Part III, p. 303). On the second day, rain fell heavily and the R.H. remained fairly constant at 95%. After 8 hr. exposure with frequent thorough stirring as before, the moisture content had risen to just

over 7%. Finally, the powder was thoroughly mixed and packed into 21 lb. cans. Fig. 1 illustrates the rate of increase of moisture content.

Thus, there were now available three batches of separated milk powder of low, medium and high moisture content, which will be denoted by the letters 'L', 'M' and 'H' respectively. 'L' was a type of powder which can readily be produced on a normal spray-drying plant when the necessary precautions are taken for obtaining a powder of low moisture content, 'M' was a powder of the kind frequently obtained under usual factory conditions, while 'H' had a moisture content as encountered in practice when powder has been stored under unsuitable conditions.

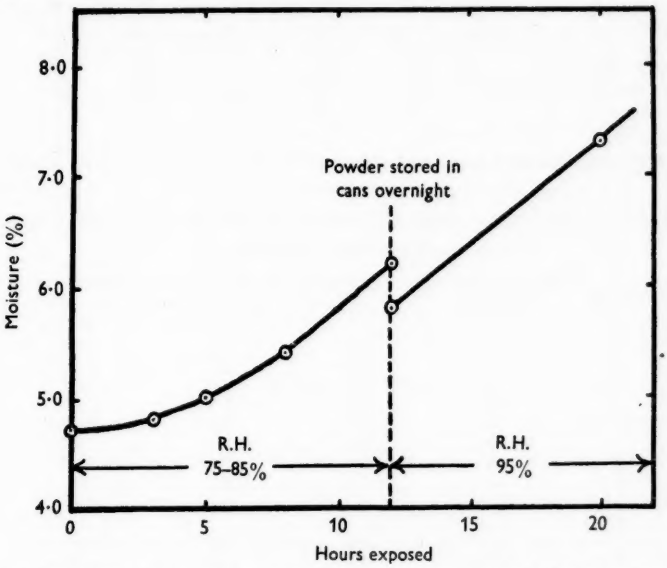

Fig. 1. The uptake of moisture by powder of 4·7% moisture content during conversion to powder of 7·3% moisture content.

Bacteriological examination of the powders by Dr C. Higginbottom indicated that no increase in bacterial population occurred during the raising of the moisture content. The technique of examination has been described previously (2). The plate counts are given in Table 1.

Table 1. *The bacterial count of the fresh powders after packing*

Powder	Plate count/g. powder	
	3 days at 37° C.	5 days at 30° C.
H	132,500	239,500
M	180,000	281,000
L	186,000	290,000

COMPOSITION AND MOISTURE CONTENT

The powders were analysed for fat by a modified Röse-Gottlieb method (3), and for protein by the usual Kjeldahl procedure. Lactose was measured polarimetrically after precipitation of the protein by mercuric nitrate or cadmium sulphate (4, 5). The analyses, which are given in Table 2, are typical of normal dried skim milks, except, of course, for the unusually high moisture content of H powder.

Moisture contents, determined by heating in an air oven for 3 hr. at 101·5° C., are given in Table 2. It is known, however, that the apparent moisture content of milk powder varies in some degree with the conditions of the determination. This variation will be particularly marked when any appreciable proportion of hydrated lactose is present, since α-lactose monohydrate, which loses very little of its 5·0% of moisture during 3 hr. at 100° C. in the air oven, is dehydrated completely in 20 hr. at 100° C. and 5 cm. pressure (6).

Table 2. *Analysis of the fresh powders*

	Powder			Moisture-free basis		
	H	M	L	H	M	L
Moisture*	7·3	4·7	2·9	0·0	0·0	0·0
Fat	1·6	1·5	0·8	1·7	1·6	0·8
Protein (N × 6·38)	32·0	32·7	34·2	34·5	34·3	35·2
Lactose† etc. (by difference)	50·9	52·9	54·2	54·9	55·5	55·9
Ash	8·2	8·2	7·9	8·9	8·6	8·1

* 3 hr. at 101·5° C.

† Lactose (anhydrous) estimated polarimetrically was 50·6, 52·8 and 53·6% for H, M and L powders respectively.

Table 3. *Moisture content of the experimental powders as determined under various conditions*

Powder	Sample no.	Hours at 100° C. (air oven)	Hours at 100° C. and 3 cm. pressure (vacuum oven)			
		3	5	10	15	20
H	1	—	7·5	7·6	7·5	—
	2	—	7·8	7·8	7·8	—
	3	—	7·7	—	—	—
	4	7·2	7·4	7·5	7·5	7·5
	5	7·2	7·4	7·6	7·6	7·6
	6	7·4	7·6	7·7	7·8	7·8
	7	—	7·4	7·4	7·5	7·5
	8	—	7·5	7·5	7·6	7·6
	Average	7·3	7·5	7·6	7·6	7·6
M	1	—	4·9	—	—	—
	2	4·7	4·9	5·0	5·0	5·0
L	1	—	2·9	—	—	—
	2	2·8	2·9	3·0	3·0	3·0

Apparent moisture contents were therefore determined on several samples of the powder after heating for 3 hr. in the air oven, or for periods up to 20 hr. in the vacuum oven at 100° C. (Table 3). Although the results do not exclude the possibility that a small proportion of the lactose had already crystallized during preparation of the powder, the greater part of the lactose must have been in the form of a non-crystalline, supercooled 'glass', such as is usually present in spray-dried milk or whey powders (7). There is a suggestion of a slight heterogeneity in the moisture figures for H powder, which will be referred to again in connexion with certain of the chemical results. Supplee (8) and Lampitt & Bushill (9) have previously stressed the difficulty of raising uniformly the moisture content of a bulk of milk powder. The equilibrium relative humidities of the three powders are given in Part III (p. 304).

Packing in air and in nitrogen

Part of each of the three powders was packed, at the Hannah Institute, in A 1 (315 ml.) tinplate 'open-top' cans, 150 g. per can, and sealed in air. Another portion of each powder was weighed into similar cans and gas-packed at Cambridge, using oxygen-free

nitrogen and a cabinet of the type previously described (10). The remainder of the powders, also gas-packed, was held at −20° C. in reserve. The seams of all cans were treated with bitumen to ensure gas tightness.

All three powders proved to be of the very rapidly desorbing type, the amount of 'entrapped' oxygen being comparatively small, and 'desorption' was practically complete within 24 hr. (Table 4). Some slight difficulty was experienced in gas-packing owing to

Table 4. *Desorption of oxygen from the powders after gas-packing*

Powder	% oxygen in the head-space gas after hours			
	0	3	24	72
H	0·1	—	1·1	1·3
M	0·1	—	1·2	1·3
L	0·1	0·9	1·6	1·6

the unusually high permeability of the particles, which permitted partial exhaustion of air from the cavities during the exhaust cycle with a consequent tendency for air to be drawn into the cans during the brief interval between removal from the cabinet and soldering of the brogue holes. This effect was minimized by holding the powder for 15 min. under a positive pressure of nitrogen before opening the cabinet. Analysis of the head-space gas in a number of the cans after gassing three times at intervals of one day showed a content of oxygen ranging from 0·0 to 0·3% with an average of 0·15%.

CONDITIONS OF STORAGE

Air- and gas-packed cans of all three powders were stored at both laboratories, the storage temperatures aimed at being 20·0, 28·5 and 37° C. At the Hannah Institute the cans were stored in incubators, the temperatures of which were checked daily. At Cambridge thermostatically-controlled rooms with rapid air circulation were used, open spacing during the first few days ensuring that the powders attained the required temperature within a very few hours. The temperatures, recorded by thermometers moved among the cans, were read daily and, over the whole period averaged well within 0·1° of the required figure, with a standard deviation from this mean of about 0·1° C. The storage experiment was commenced at the same date in both laboratories, and the powders examined thereafter at intervals as described in Parts III, IV and V.

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PART III. PHYSICAL, CHEMICAL AND PALATABILITY CHANGES IN THE STORED POWDERS

By C. H. LEA and J. C. D. WHITE

(With 22 Figures)

As already described in Part II separated milk powders of three moisture contents, packed in air and in nitrogen, were stored at three temperatures at both the Low Temperature Research Station and the Hannah Dairy Research Institute, and sample cans removed at intervals for examination by taste and by various physical and chemical methods. At a comparatively early stage of the work it became apparent that appreciably different rates of deterioration of the least stable powder at the highest storage temperature were being observed at the two laboratories, differences which were probably due to minor variations in effective storage temperature coupled with a very high temperature coefficient of some of the reactions involved in deterioration. Therefore, as a precautionary measure, some of the chemical determinations were duplicated at the two laboratories. In such cases one set only of the results has been presented in detail, the other being given in summarized form.

Changes in palatability

The necessity for carrying out tasting tests in experimental work on the keeping properties of milk powder, and some of the weaknesses inherent in the tasting panel technique have previously been discussed in connexion with whole-milk powder(1). In order to obtain evidence as to the magnitude of these difficulties, and at the same time to reduce their effect, the system devised for use with whole-milk powder was extended to separated powder. According to this scheme the various series of deteriorated powders were examined independently at the two laboratories, each choosing its own technique, and a comparison of the results was made only at the end of the storage experiment.

Technique

In the method used at Cambridge the stored powders were reconstituted in distilled water at 20° C. in proportions corresponding to 10 g. dry milk solids per 100 ml. water. The samples, labelled in a code unknown to the tasters, were graded half an hour later by a panel of six persons, marks being awarded according to the scale: 6 = very good, 5 = good, 4 = fairly good, 3 = fair, 2 = rather poor, 1 = poor, 0 = very poor, as previously used for full-cream powders(1, 2). While the attainment on this scale of a score of 4 is usually considered to mark the end of the life of a powder as a product of reasonably good quality for household use, no large-scale consumer trials were carried out and the function of the numerical scale employed in these experiments was mainly to facilitate comparison between the various treatments. The standard adopted was fairly severe; the powders scoring 3 or even 2 would probably be quite usable for some manufacturing or domestic cooking purposes.

In the method used at the Hannah Institute the powders were coded, reconstituted in distilled water at 35° C. using a powder to water ratio of 1 : 10, and tasted within 30–60 min. by a panel of five persons. A powder of good quality was included in each tasting as a standard or control powder. The tasting and scoring technique has been described previously with reference to whole-milk powders(1, 2), but the scoring system is briefly as follows: 0 = very good and very like ordinary fresh milk, 1 = fairly good and

quite palatable, 2 = slightly but definitely unpalatable due to the presence of slight 'off'-flavours, 3 = unpalatable due to the presence of pronounced 'off'-flavours, 4 = very unpalatable. This method of scoring measures the '*un*palatability' of a powder and the average of the 'off'-flavour marks awarded to it by the five tasters is termed its 'off'-flavour score. When a powder passes 'off'-flavour score 1·0, the trained panel is beginning to detect the development of slight 'off'-flavours. When score 2·0 is reached, the 'off'-flavour would probably be noticed and objected to by the ordinary consumer.

H, M and L powders tasted prior to storage against separated fresh milk (flavour score 6·0, 'off'-flavour score 0·0) were awarded scores of 5·2, 5·6 and 5·5, and 0·4, 0·2 and 0·1 respectively according to the two systems. It was obvious that H powder had already commenced to deteriorate in flavour during the short interval of approximately 10 days occupied by rail transport, etc., between preparation and beginning of the storage

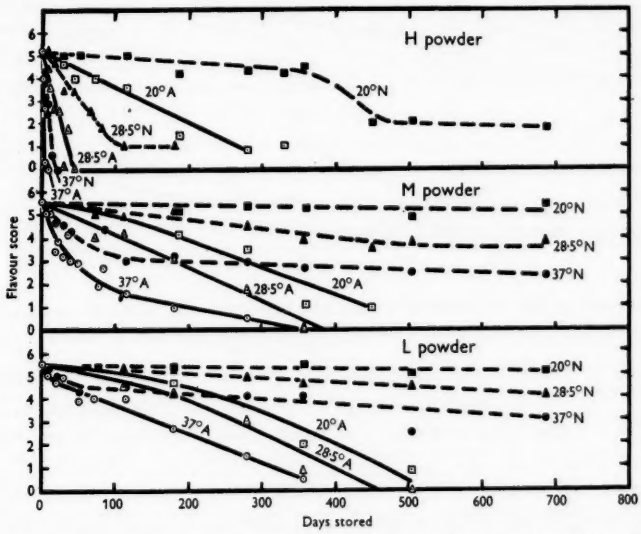

Fig. 1. Changes in flavour of the air-packed and nitrogen-packed powders during storage at 37, 28·5 and 20° C. (Cambridge results).

experiment. Thereafter only M or L powder, gas-packed and held at −20° or at 0° C. was used as control in the grading of powders stored at higher temperatures.

The results of the storage experiments, which are given in Figs. 1 and 2, and in Table 1, may be summarized as follows.

Effect of moisture content

The most noteworthy feature of the results was the great rapidity of deterioration of H powder, as compared with M and L powders. Storage life, as determined by measurement of palatability, ranged from about 2 days for H powder packed in air and stored at 37° C. to periods of the order of 2 years or longer for powders of low and medium moisture content packed in nitrogen and stored at 20° C.

The higher the storage temperature the greater was the deleterious effect of a high moisture content. In the air-stored series the ratios of the storage lives of the H, M and L powders, as estimated by the Cambridge panel from the average of the data for flavour

scores 4, 3 and 2, were of the order of 1 : 16 : 50 at 37° C., 1 : 10 : 15 at 28·5° C. and only 1 : 2·1 : 2·6 at 20° C. (cf. Table 1). The corresponding ratios as estimated by the Hannah Institute panel were even higher, 1 : 58 : 87 at 37° C., 1 : 21 : 30 at 28·5° C. and 1 : 3·5 : 4·1 at 20° C. Data for the gas-stored powders are incomplete.

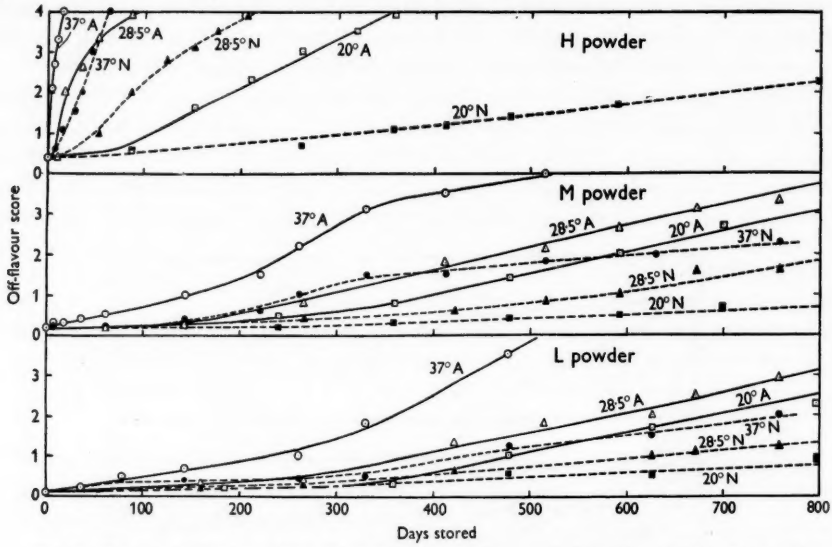

Fig. 2. Changes in flavour of the air-packed and nitrogen-packed powders during storage at 37, 28·5 and 20° C. (Hannah Institute results).

Table 1. *Deterioration in palatability of skim-milk powder on storage**

		Cambridge results: Time taken to deteriorate to flavour score (days)						Hannah Institute results: Time taken to deteriorate to 'off'-flavour score (days)					
		Air-pack			Nitrogen-pack			Air-pack			Nitrogen-pack		
Powder	Storage temp. (° C.)	4	3	2	4	3	2	1	2	3	1	2	3
H	37	2	3	4	5	8	10	2	4	8	18	34	49
	28·5	10	18	27	30	55	78	12	21	44	51	92	140
	20	75	140	200	380	430	480	115	180	270	340	*c.* 690	—
M	37	20	44	88	50	200	—	145	250	320	260	*c.* 660	—
	28·5	110	180	250	400	—	—	290	480	*c.* 660	590	—	—
	20	190	270	360	—	—	—	410	590	—	—	—	—
L	37	80	160	240	250	700	—	240	350	430	450	—	—
	28·5	190	270	330	—	—	—	370	590	—	*c.* 650	—	—
	20	250	330	400	—	—	—	500	*c.* 670	—	—	—	—

* Absence of a figure in the table signifies a period of over 700 days.

Temperature coefficients

The temperature coefficient of the reactions responsible for deterioration of flavour in H powder was unusually high, an increase in rate of spoilage of the order of sixfold or more resulting from an increase in storage temperature from 20 to 28·5° C., or from 28·5 to 37° C. This was the case for the Cambridge results whether storage was in air or in inert gas. A similar factor was found by the Hannah Institute panel for air-stored powder over both temperature ranges, and for gas-stored powder over the lower range; between 28·5 and 37° C. in nitrogen a rather lower factor of approximately 3 was obtained.

The effect of temperature on the deterioration of M and L powders was very much less marked. For M powder the acceleration factor averaged only 1·5 for both panels over the range 20–28·5° C., and 2·0 (Hannah Institute) or of the order of 5 (Cambridge) for the range 28·5–37° C. For L powder the corresponding factors averaged only 1·2 and 1·6 for the two ranges.

It may be concluded that in powder of high moisture content, and perhaps even to some extent in powder of medium moisture content at high storage temperatures, changes resulting in spoilage of flavour occur which do not take place to any appreciable extent in powder of low moisture content even at 37° C. The nature of these changes will be discussed later (p. 335).

Effect of gas-packing

Packing in an atmosphere of nitrogen considerably delayed deterioration in flavour, the average 'protection factor' for all powders, storage temperatures and degrees of 'off'-flavour, as estimated by the Cambridge panel, being about 3. The corresponding factors recorded by the Hannah Institute panel were of the order of 2 for L and M powders, 3 for H powder at 20 and 28·5° C., and as much as 8 for H powder at 37° C. (Table 1). The advantages of nitrogen-packing, which can be seen more clearly in Figs. 1 and 2, suggest that in powders of all moisture contents part at least of the 'off'-flavours produced must result directly or indirectly from reactions involving atmospheric oxygen.

Types of 'off'-flavour

Two main types of 'off'-flavour were detected. In the air-packed series, particularly at high moisture content, the powders commenced by developing a 'stale' or 'cardboard' flavour which gradually intensified to a characteristic nauseating and very unpleasant 'gluey' taste. In air-packed powders of low moisture content 'off'-flavours developed very much less rapidly and were less obviously 'glue-like' in character. Since a recognizably tallowy flavour has previously been observed to develop in air-packed separated milk powder containing 2·4% of fat (3), it is quite possible that oxidation of the rather smaller proportions of residual fat present in these powders (cf. Part II, Table 2) may have made a significant contribution to deterioration over long periods of storage, although a tallowy flavour could not be identified specifically.

The nitrogen-packed powders did not develop the same type or degree of unpalatability on storage. Their typical 'off'-flavour tended to be a 'heated', 'cooked', 'caramelized' or slightly 'stale' flavour, suggestive of evaporated milk. A long-stored, gas-packed high moisture powder might have a very poor solubility, a brownish colour, and show an abnormal degree of frothing* on reconstitution, yet, its predominantly 'caramelized' flavour, though far removed from that of fresh milk, could not be considered really unpleasant. Gas-packed powders of low moisture content showed very little deterioration in flavour during the period of the experiment.

Since the flavour of air- and nitrogen-packed powders so frequently differed markedly in quality as well as in degree, it was inevitable that different groups of observers would differ in their estimates of the magnitude of the improvement in palatability or in storage life effected by packing in inert gas. A sufficient measure of agreement was, however,

* Such frothing is known to be a property of the 'melanoidins' formed by the interaction of amino-acids or protein and reducing sugars.

registered between the two panels to establish the usefulness of gas-packing for preserving the palatability of separated milk powder, as had previously been done for whole-milk powder (3).

Changes in colour

For the measurement of colour, which was carried out at both laboratories, the powders were packed in a standardized manner into small porcelain dishes and examined in the Lovibond Tintometer with artificial light attachment. At Cambridge, the Lovibond-Schofield modification of the instrument was used, in which illumination was by C.I.E. Standard Illuminant B, consisting of gas-filled metal filament bulbs operating at a colour temperature of 2848° K., used in combination with the specified colour filter solutions.

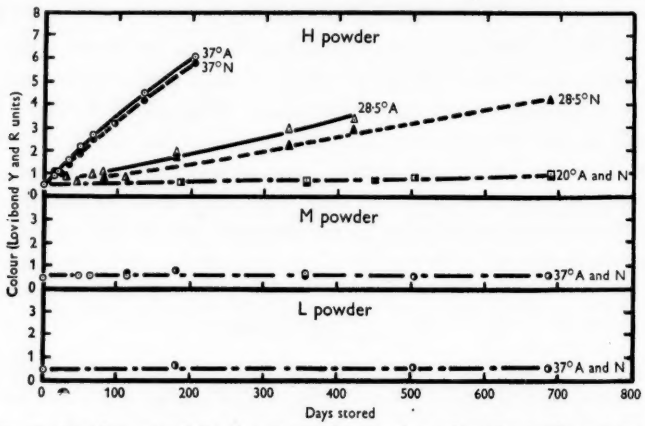

Fig. 3. Development of discoloration in the stored powders (Cambridge results).

Table 2. *Rates of discoloration of skim-milk powder during storage*

		Increase of discoloration (Lovibond units/100 days)			
		Cambridge results		Hannah Institute results	
Powder	Storage temp. (° C.)	Air-pack	Nitrogen-pack	Air-pack	Nitrogen-pack
H	37	3·0	2·8	2·6	2·2
	28·5	0·7	0·6	0·7	0·6
	20	0·0 (7)	0·0 (6)	0·0 (5)	0·0 (3)
M	37	0·0 (2)	0·0 (2)	0·0 (3)	0·0 (3)
L	37	0·0 (0)	0·0 (0)	0·0 (2)	0·0 (2)

Colour quality in both instruments was matched by standard yellow and red glass slides, while the relative brightness of the sample and of the comparison field could be equalized by means of the obturator vane fitted to the light cabinet, or by the use of neutral tint slides. In practice it was found that little use of these latter controls was necessary over the limited range of brightness encountered in the storage experiments, and, for simplicity, colours have been recorded simply as the sum of the yellow and red units used.

Reproducibility in the measurement of colour by this means was not of a very high order, and different operators were liable to obtain appreciably different results. The figures obtained, however, indicate sufficiently clearly the order of magnitude of the effects of the various factors under investigation on the rate of discoloration of separated milk powders (Fig. 3, Table 2).

As with the majority of the other characteristics measured, high moisture content and high storage temperature were the factors chiefly concerned in causing spoilage. The progress of discoloration was very roughly linear, the air-packed powders changing just perceptibly more rapidly than those in nitrogen. Relative rates of discoloration of H powder at 20, 28·5 and 37° C. were of the order of 1 : 13 : 53 (Table 2). The very high temperature coefficient between 20° and 28·5° C. and the slight induction period visible at the latter temperature are probably due, in part at least, to an increasingly delayed crystallization of lactose as the storage temperature is reduced (cf. p. 305).

Changes in the colour of M and L powders, even after 2 years at 37° C., were very slight.

CHANGES IN pH (J. C. D. WHITE)

The powders were reconstituted in distilled water to give a 9% solution of milk solids, and the hydrogen-ion concentration measured by glass electrode and pH meter. The results (Fig. 4) show that, as with the development of colour, high moisture content and

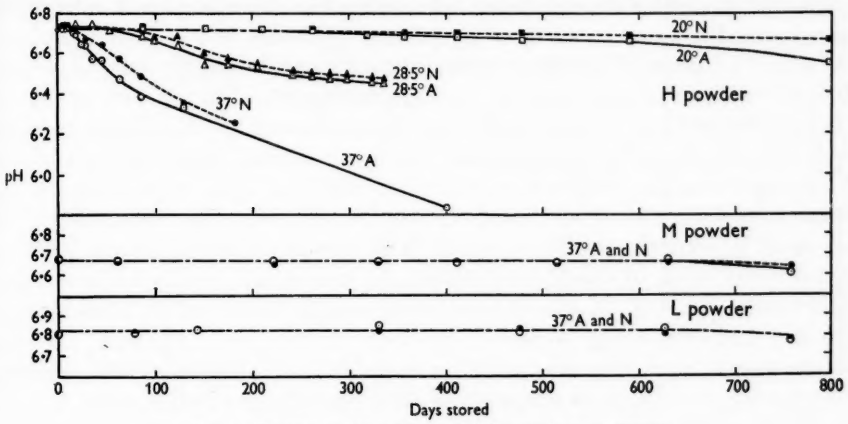

Fig. 4. Changes in the pH value of the powders on storage.

high storage temperature were the factors mainly concerned in producing a change in pH: after 400 days at 37° C. the pH of reconstituted H powder had fallen from 6·72 to 5·83. Packing in nitrogen slightly retarded, but did not prevent, the decrease. M and L powders retained their initial pH values almost unchanged, even after storage for 2 years at 37° C.

Further data on pH changes in H powder, and evidence bearing on the cause are given in a subsequent section (p. 318).

CRYSTALLIZATION OF LACTOSE, AND CHANGES IN EQUILIBRIUM RELATIVE HUMIDITY (C. H. LEA)

Lactose exists in spray-dried milk powder as a supercooled, highly concentrated solution or 'glass' composed of α- and β-sugars in the ratio of about 1 : 1·5, which is stable for long periods at normal temperatures and low relative humidities. If the moisture content of the powder is sufficiently high, or if a dry powder is permitted to absorb sufficient water vapour from the atmosphere, α-lactose monohydrate, which contains 5·0% of water, crystallizes out on storage, and the powder 'cakes', the change being greatly

accelerated by high storage temperatures. At sufficiently high relative humidities (e.g. 70%) β-lactose is slowly converted to the crystalline α-hydrate (4).

In the present experiments H powder, after storage at 37° C. for a few days, set to a solid mass in the can, indicating extensive crystallization of the lactose. This conclusion was confirmed by observation under the polarizing microscope, and by seeding tests with a supersaturated lactose solution (4). It was not, however, found practicable to make either of these tests quantitative.

Since it was felt that the influence of moisture on chemical or physical changes occurring in the powder during storage would be more closely related to the activity of water as indicated by relative humidity measurements than to moisture content as determined by one or other of the empirical methods available, equilibrium relative humidity measurements were carried out as follows.* Several 1 g. portions of each sample of powder were weighed into light aluminium dishes 2 in. in diameter, and each dish was suspended over a solution of sulphuric acid of suitable concentration in a closed, 'Kilner' type glass jar. The wire supporting the dish passed between the two halves of a small, split rubber stopper inserted in a hole in the lid of the jar and terminated in a loop for attachment to the arm of a balance. With this arrangement each sample of powder could be weighed at intervals without removal from its particular atmosphere. The jars were stored and the samples weighed in a thermostatically controlled room at 20° C. The relative humidity at which the sample neither gained nor lost weight, as obtained from a plot of the gains and losses at the various relative humidities used, was taken as the equilibrium R.H. The determination was not considered satisfactory unless sulphuric acid solutions within 5% above and 5% below the equilibrium value for the powder under examination had been included in the series. It should be noted that, for convenience, equilibrium R.H. values were determined at 20° C. for all milk powder samples, irrespective of the temperature at which they had been stored. Values for the fresh powders were: H powder, 7·5–7·8% moisture, 41–43% R.H.; M powder, 5·0% moisture, 29% R.H.; L powder, 3·0% moisture, 17·5% R.H. There was again a suggestion of slight heterogeneity in H powder.

In Fig. 5, equilibrium R.H. values for the various stored powders are plotted against storage time, each point being representative of the contents of an individual can. In order to examine more closely the first portion of the curve for H powder at 37° C., 5 g. portions of control powder from the well-mixed contents of a single can which had been held under nitrogen at −20° C. were sealed in glass tubes, heated rapidly to 37° C. in a water-bath, and stored at 37° C. The superior reproducibility and slightly higher equilibrium value after crystallization obtained under these conditions again suggest a slight lack of homogeneity in the large bulk of H powder used in the main experiment.

Crystallization of all the α-lactose originally present in the supercooled 'glass' form would absorb only about 1·1% of the 7·5–7·8% of moisture originally present in H powder. Lactose as concentrated syrup or 'glass' is much more hygroscopic than when crystalline. Crystallization of α-lactose hydrate would therefore cause dilution of the remaining lactose glass (some 60% of the original total) and tend to cause an increase in equilibrium R.H. The observed increase in this value from *c.* 42 to *c.* 55% on storage of H powder can therefore be explained qualitatively by the crystallization of lactose.

After a relative humidity of about 55% had been attained there appeared to be no further increase on continued storage despite the fact that a gradual conversion of β-

* The method described is an adaptation of that used by Gane (*J. Soc. chem. Ind., Lond.* (1941), **60**, 44) for investigation of the water relations of wheat.

lactose to α-lactose hydrate was still taking place (p. 306). Presumably the falling concentration of 'free' water in the powder and the hygroscopic nature of the residual protein combined to prevent a further rise in equilibrium relative humidity beyond this point.

The experimental results (Table 3) show that lactose commenced to crystallize in H powder only after a marked 'induction period' of the order of 1 day at 37°, 10 days at 28·5° or 100 days at 20° C. Crystallization was slightly delayed in nitrogen as compared with air, but the difference due to this cause was small at 37 and 28·5° C., the difference at 37° C. quoted in the table including the effect of a slower attainment of thermostat temperature by the powder in the large, gas-packed cans as compared with the small, air-packed tubes.

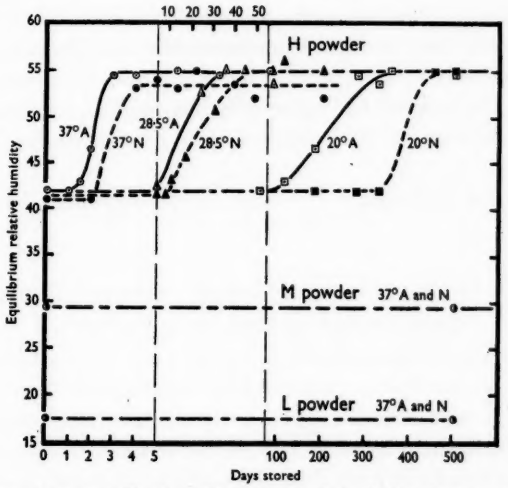

Fig. 5. The effect of the crystallization of α-lactose hydrate during storage on the equilibrium relative humidity of the powders.

Table 3. *The crystallization of lactose in separated milk powder of 'high' (7·5–7·8%) moisture content during storage*

Storage temp. (° C.)	Atmosphere	Container	Delay in onset of crystallization (days)	Increase in relative humidity complete after (days)
37	Air	Small glass tubes	1	3
37	Nitrogen	Cans	2	4
28·5	Air	Cans	5	30
28·5	Nitrogen	Cans	10	40
20	Air	Cans	100	300
20	Nitrogen	Cans	300	400

At 20° C., gas-packed H powder commenced to crystallize some 6 months later than the air-packed material, a delay which was reflected in the very appreciably slower rate of deterioration shown by this powder when judged by several different criteria (cf. Figs. 13, 16–18). Since the 'gas powder' was packed at Cambridge and the 'air powder' at the Hannah Institute, part of both packs then being stored at each laboratory, it is just possible that the air-gas difference observed might have been due to a very slight initial difference in the moisture content of the powder packed at the two laboratories, or to a small loss of moisture during the exhaust stage of the multiple gas-packing technique used (Part II, p. 296). It should be stated, however, that careful comparison of the

total moisture content of one sample of 20° C. H air-packed powder which had crystallized, with a sample of 20° C. H gas-packed powder of the same age which had not crystallized failed to show any appreciable difference in total moisture content. On the other hand, several cans of 20° H air-packed powder of the same age, which had been selected by equilibrium R.H. determination and by analysis of the headspace gas as having commenced to crystallize at different dates, showed moisture contents varying over a small range in precisely the expected manner. The balance of the evidence available therefore suggests that some cause other than slight fortuitous variation in moisture content may be responsible for the delayed crystallization of lactose in gas- as compared with air-packed H powders.

The possible influence of factors such as agitation in starting crystallization has not been explored. The data given in Fig. 5 show the condition of the particular can, or group of cans, of H powder used for the chemical investigations. Samples of powder removed from a can of which the contents had apparently just commenced to crystallize, repacked in glass tubes and held at 20° C. completed their rise in relative humidity in about 1 month.

M and L powders had not begun to crystallize even after storage for as long as 600 days at 37° C.

Conversion of β-lactose to α-lactose hydrate and decrease of total soluble lactose (J. C. D. White)

Troy & Sharp (4) observed a considerable change of β-lactose to α-lactose hydrate in milk products stored at laboratory temperature and 70% relative humidity for 5 months. Since the initial high equilibrium R.H. (*c.* 42%) of H powder gradually increased to *c.* 55% because the anhydrous α-lactose crystallized to the hydrated form, it was thought probable that, in this powder at least, β-lactose would be converted to α-hydrate, especially at the higher storage temperatures.

A polarimetric method of estimating the proportions of α- and β-lactose in milk products has been described by Sharp & Doob (5). To gain experience of the method, it was applied to fresh liquid milk and the proportions of α- and β-lactose found (38·5% α, 61·5% β at 25° C.) were very close to the β/α equilibrium ratio of 1·58/1 (38·7% α, 61·3% β) for that temperature. Examination of the fresh H, M and L powders showed that 41–43% of the lactose was in the α form. As Troy & Sharp (4) have pointed out, this higher percentage of α-lactose is to be expected since the milk would be dried at a temperature above 25° C., so decreasing the β/α ratio.

Air- and nitrogen-packed samples of each powder were examined after different periods of storage. Sharp & Doob (5) advocate the use of 'norrit' as a decolorizing-agent when examining brown, deteriorated samples, especially of dried whey, but this was not found necessary with the milk powders. The results (Fig. 6) show that in air- and gas-packed M and L powders, the proportions of α- and β-lactose were unchanged even after more than 600 days' storage at 37° C. In H powder, however, β-lactose was converted to α-lactose, presumably the hydrated form, fairly rapidly at 37° C. and more slowly at 28·5 and 20° C. until after 400 days at 37° C., 98·5% of the lactose was in the α-form.

The H samples were examined after they had been taken from the incubators and stored in bottles at 0–4° C. for some time, so that any differences in the rate of conversion of β- to α-lactose due to gas-packing may have been minimized. Nevertheless, as in the

crystallization of the anhydrous α-lactose, the absence of oxygen slowed the change, although this effect was probably due simply to the slower rise in the equilibrium R.H. of the gas-packed powders.

Sharp & Doob's method(5) also measures total lactose but they have shown that the protein precipitant used, an alcoholic solution of mercuric chloride, causes a slightly low value for the apparent percentage of total lactose. However, it was obvious that during storage of H powder there was a progressive decrease in the amount of total soluble lactose. After 400 days at 37° C., the percentage of total lactose, as estimated by this method, had decreased from 50·3 to *c.* 44%. With three other protein precipitants, the total soluble lactose averaged 45·3%. The reason for this decrease is discussed fully in a later section (p. 322).

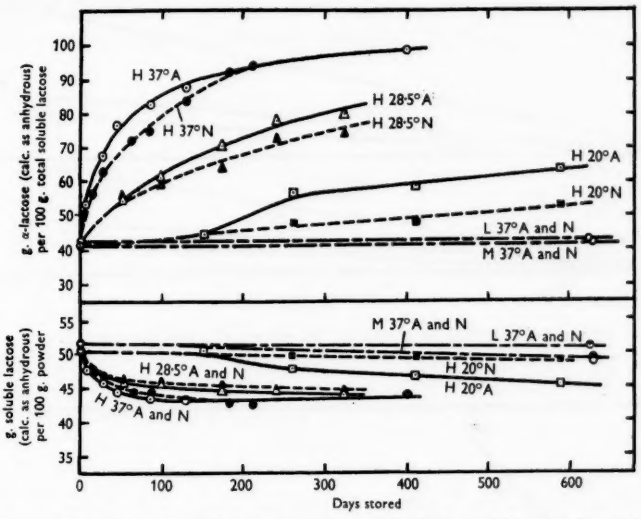

Fig. 6. The conversion of β-lactose to α-lactose hydrate, and decrease in total soluble lactose during storage.

ABSORPTION OF OXYGEN AND PRODUCTION OF CARBON DIOXIDE

As previously stated (Part II, p. 296), the powders were packed in gas-tight cans of 315 ml. capacity at the rate of 150 g. per can which, under the conditions of sealing, corresponded to a free oxygen content in the air- and gas-packed cans of 39·2 and 0·3 mg./100 g. powder respectively. For the purpose of this calculation the density of air-free separated milk powder was assumed to be 1·465(6). Prior to opening for examination a sample of the head-space gas was withdrawn from each can of stored powder by the technique previously described(3), and submitted to analysis for oxygen and carbon dioxide in a Haldane apparatus. The results, converted to absolute units by a calculation based on the constancy of the nitrogen content of the can, are summarized in Figs. 7 and 8.

Absorption of oxygen and production of carbon dioxide by H powder commenced with a marked 'induction period' of the order of 2, 20 and 200 days at 37, 28·5 and 20° C. respectively, which probably corresponded to the periods required for the crystallization of lactose under these conditions (cf. Table 3). Thereafter the rate of gas exchange was

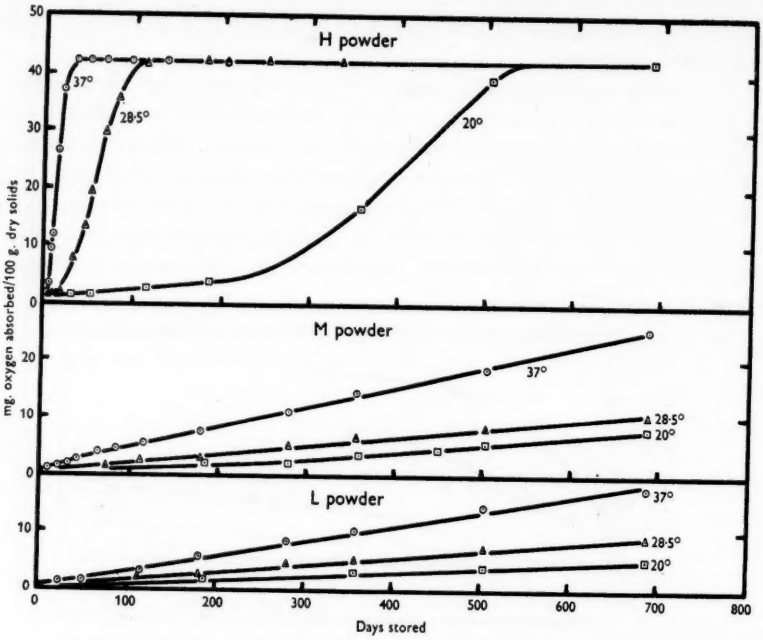

Fig. 7. Absorption of oxygen by the powders (Cambridge results).

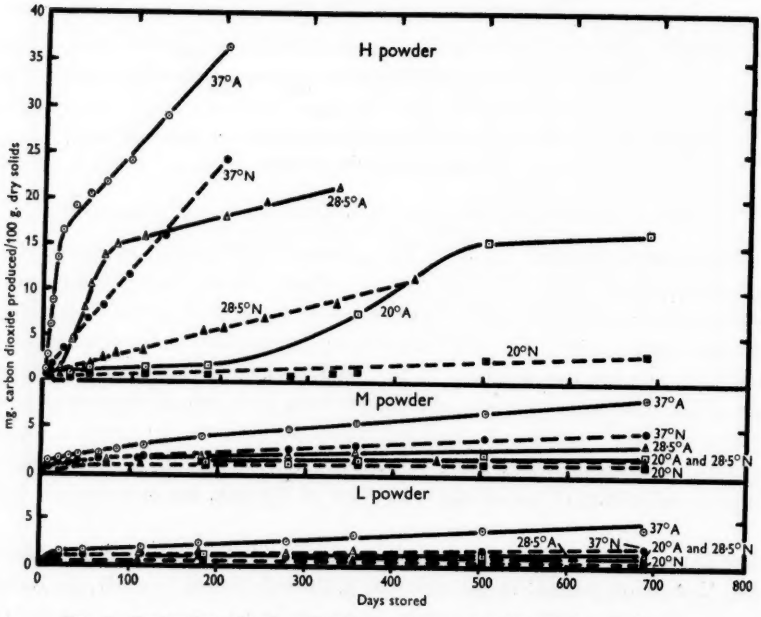

Fig. 8. Production of carbon dioxide by the powders (Cambridge results).

rapid, production of carbon dioxide being nearly ten times more rapid by air-packed than by gas-packed powders until all free oxygen in the container had been exhausted, after which the lower rate became common to both powders.

Absorption of oxygen and production of carbon dioxide by M and L powders were very much slower, and the curves were almost linear except for a small initial excess production of carbon dioxide (<1 mg./100 g. powder) which was possibly due to equilibration of gas within and between the particles or to the decomposition of some labile minor constituent. The gradients of these lines are given in Table 4, together with approximate estimates of the corresponding values for H powder.

As with flavour, the increase in rate of gas exchange in passing from 3·0 or 5·0 to 7·6% moisture content was very great, and was greater the higher the storage temperature. The acceleration of deterioration which occurred after the commencement of crystallization of the lactose in H powder was very marked.

Table 4. *Rates of absorption of oxygen and production of carbon dioxide by the powders*

	Absorption of oxygen or production of carbon dioxide (mg./100 g. milk solids/100 days)								
	H powder			M powder			L powder		
	37° C.	28·5° C.	20° C.	37° C.	28·5° C.	20° C.	37° C.	28·5° C.	20° C.
Cambridge results									
Oxygen absorption in air-pack:									
Main reaction	250	55	15	3·5	1·5	0·9	2·5	1·2	0·7
Carbon dioxide production in air-pack:									
Main reaction	100	24	6	1·0	0·3	0·2	0·4	0·1	<0·1
After disappearance of free oxygen	11	2·5	0·5	—	—	—	—	—	—
Carbon dioxide production in gas-pack:									
Main reaction	11	2·5	0·5	0·5	0·2	<0·1	0·2	<0·1	<0·1
Hannah Institute results									
Oxygen absorption in air-pack:									
Main reaction	240	45	10	3·0	1·5	1·0	2·5	1·3	0·7
Carbon dioxide production in air-pack:									
Main reaction	96	18	6	0·7	0·3	0·2	0·4	0·1	0·1
After disappearance of free oxygen	9	2·0	?	—	—	—	—	—	—
Carbon dioxide production in gas-pack:									
Main reaction	9	2·6	?	0·4	0·2	<0·1	0·2	<0·1	<0·1

Supplementary experiment

The supply of free oxygen available in the air-packed cans of H powder had become nearly exhausted after 20 days, and completely exhausted after about 30 days, at 37° C. Subsequent storage was therefore under conditions which approximated to those in the gas-packed containers.

Since in commercial practice containers used for air-packing milk powder are not likely to be completely gas tight a small supplementary experiment providing a larger supply of oxygen (141 mg./100 g. of powder) was undertaken, using H powder only. After 60 days at 37° C. the concentration of oxygen in the cans had fallen from 20·9 to 2·5%, corresponding to the absorption of 128 mg. of oxygen per 100 g. of powder. (Part of this 'extra air' material was used in one of the rat-feeding tests described in Part IV (Exp. 8).)

Changes in solubility

Estimation of soluble solids

The solubility of the stored powders in cold (20 C.) and hot (50 or 60° C.) water was estimated at intervals at both laboratories by modifications of the method of Howat, Smith, Waite & Wright (7). In this method the powder is reconstituted with distilled water at a powder to water ratio of 1 : 9, the insoluble material is centrifuged down, and the solids content of the supernatant liquid is measured. The ratio of the dissolved solids to the total dry solids initially present, expressed as a percentage, is taken as an index of the solubility of the powder.

It had previously been observed with very soluble powders that solubility indices determined at the Hannah Institute by this method frequently exceeded the theoretical maximum of 100 by 2–3 units, an anomaly which was considered as probably due mainly to partial hydration of originally anhydrous lactose during the determination (3). It has

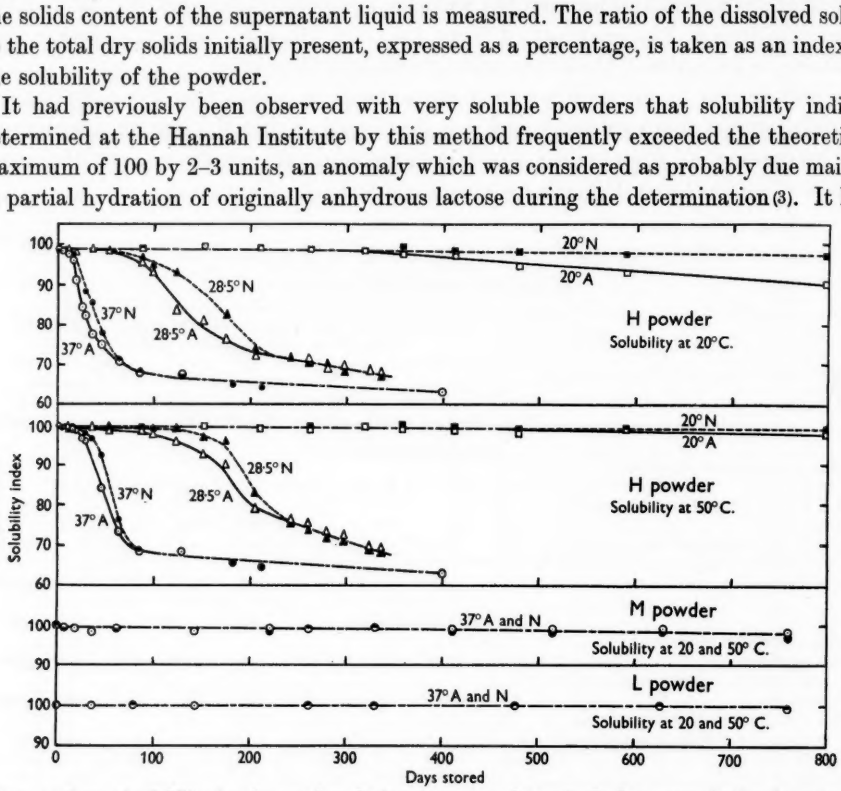

Fig. 9. Loss of solubility by the powders during storage, as determined after reconstitution in water at 20 and at 50° C. (Hannah Institute results).

therefore been usual to apply a small correction to the experimentally determined figures to allow for this error (1, 2), a procedure which has been followed at the Hannah Institute in the present work.

In the slightly different procedure adopted at Cambridge a control determination of dry weight, carried out on the reconstituted powder without centrifuging, was run side by side in each determination with that on the centrifuged sample, in order to supply the necessary correction. The deviation from 100% solubility found in the controls ranged from about 0·2 to 1·0, with a mean of about 0·5%, indicating that under the particular conditions of drying used the degree of hydration of the lactose occurring was comparatively slight.

The solubility of the fresh powders in water at 20 and at 50 or 60° C. approximated to 99 and 100% respectively. The results obtained on storage of the three powders in air

and nitrogen at 37, 28·5 and 20° C. are shown graphically in Fig. 9, while the times taken by the powders to reach various stages of insolubility are listed in Table 5. Reduction of the solubility of the powder to *c.* 65% implies complete insolubility of the protein fraction. The data show that the moisture content of the powders or, more correctly, the activity of water in the powders, exercised a decisive influence on the development of insolubility, H powder showing a greater loss of solubility after storage for 1 week at 37° C. than M or L powder after 2 years at the same temperature.

The temperature coefficient for this form of deterioration was again high, an average factor of about 5 being observed for the temperature interval 37–28·5° C., with signs of an even greater factor between 28·5 and 20° C. (Table 5). Delayed crystallization of the lactose with its accompanying increase in equilibrium R.H. (cf. Fig. 5, Table 3) probably contributed to the induction period, which was of the order of 5–10 days at 37°, 40–80 days at 28·5° and 400 days or longer at 20° C., and which preceded any loss of solubility in the

Table 5. *Loss of solubility of H powder during storage**

(Initial values 99% at 20° C.; 100% at 50 or 60° C.)

Storage temp. (° C.)	Pack	Time taken to deteriorate to the stated percentage solubility (days): In water at 20° C. 95%	90%	80%	70%	In water at 50 or 60° C.† 95%	90%	80%	70%
		Hannah Institute results							
37	Air	16	20	28	70	28	36	52	74
	Nitrogen	20	28	41	70	39	48	58	78
28·5	Air	86	105	150	260	130	165	200	310
	Nitrogen	100	135	180	260	170	190	220	310
20	Air	500	—	—	—	—	—	—	—
	Nitrogen	—	—	—	—	—	—	—	—
		Cambridge results							
37	Air	9	12	18	55	22	28	38	64
	Nitrogen	11	14	22	55	25	32	41	65
28·5	Air	52	68	110	240	100	140	210	320
	Nitrogen	65	82	130	260	120	160	230	340
20	Air	450	610	—	—	—	—	—	—
	Nitrogen	620	—	—	—	—	—	—	—

* Absence of a figure in the table signifies a period of over 700 days.

† Solubility in hot water was measured at 50° C. at the Hannah Institute, and at 60° C. at Cambridge.

H powder. On the other hand, later experiments with artificial dialysed protein-sugar systems stored at a constant relative humidity also showed a marked induction period (8), which indicates that the crystallization of lactose cannot have been the main factor in delaying the onset of loss of solubility of the milk powders. This point is treated more fully in a subsequent section (p. 332).

Insolubility in water at 50 or 60° C. developed much later than insolubility in water at 20° C., and can be considered as representing a more advanced stage of deterioration (Fig. 9, Table 5). Partly deteriorated samples which, although of poor solubility in cold water, still remained quite soluble in hot, thus displayed an interesting similarity to roller-dried powder in which some protein insolubility has been caused by severe heat treatment during drying. Wright (9) has shown that the difference in solubility of roller-dried powders when estimated at 20 and 50° C. is a measure of the amount of protein rendered insoluble by dry heat. Whether or not the mechanism of deterioration by excessive heat during and immediately after removal of most of the water in the roller-

drying process is the same as that produced by storage of powder at too high a moisture content and storage temperature is not yet known, and requires investigation.

The gas-stored samples developed insolubility definitely more slowly than the air-stored samples but the differences were never very great and can be accounted for, in part, at least, by the slightly delayed crystallization of lactose in the gas-stored powders.

Table 5 discloses a considerable difference in the rates at which powders stored at the two laboratories, ostensibly under the same conditions, developed insolubility. The effect seems too large to be accounted for entirely by slight differences in effective storage temperatures, and is probably due in part to the different mechanical means employed at the two laboratories to reconstitute the powders. It is well known that the degree of stirring or shaking to which a milk powder is subjected during reconstitution markedly influences its apparent solubility (7).

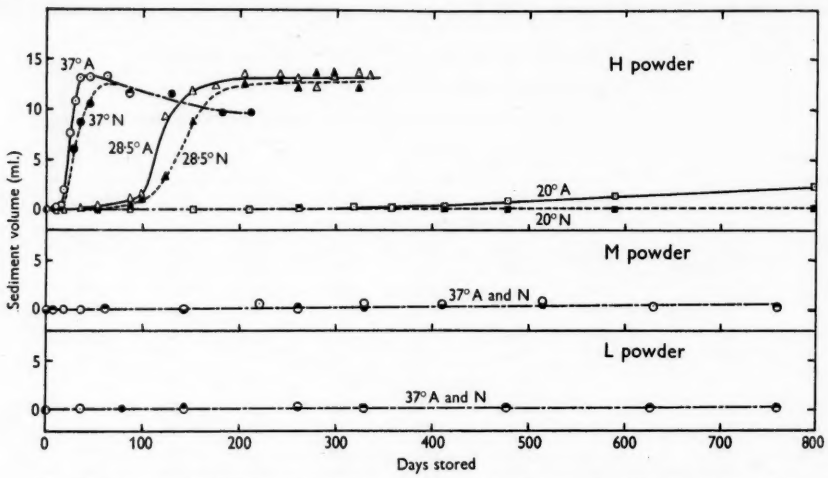

Fig. 10. Loss of solubility by the powders as measured by increase in sediment volume after reconstitution in water at 20° C. (Hannah Institute results).

Measurement of sediment volume

The American Dry Milk Institute Inc. has developed a standard 'sediment' solubility method, but as the most recent description of this method (10) specifies apparatus which was unobtainable at the time, the procedure was altered slightly. The modified method, as used at the Hannah Institute, was as follows: 20 g. powder were added to 200 ml. distilled water at 20° C. contained in a 500 ml. wide-mouthed bottle. (Before adding the powder, the bottle was shaken to wet the sides and prevent the powder sticking to the bottle.) The bottle was shaken for 30 sec., allowed to stand for 5 min., shaken again for 30 sec. and a centrifuge tube of 50 ml. capacity filled to the mark with the 'milk'. The tube was then centrifuged for 15 min. at 1000 r.p.m. (216 *g*). The supernatant liquid was removed by suction, 25 ml. water at 20° C. added and the tube shaken for 30 sec., taking care to dislodge all the sediment. The tube was filled to the mark with water at 20° C. and finally centrifuged for 15 min. as before. The volume of the sediment thus obtained gave a measure of the solubility. The results obtained by the application of this method to the stored powders are shown in Fig. 10.

These estimations bore out the conclusions obtained by the more absolute method. It was found that the volume of sediment obtained from the H powder at 37° C. decreased after the powder had deteriorated to its maximum insolubility (i.e. as judged by sediment volume). This decrease was probably due to some physical change in the coagulated protein particles which enabled them to 'pack' more closely when centrifuged. It was also noticed with severely deteriorated H powder, that some insoluble material was present on the surface of the supernatant liquid after centrifuging. When this layer was broken up and the suspension recentrifuged, the amount of floating material was reduced but could not be removed completely. The trace of fat present may have carried up insoluble protein to the surface and this was probably a minor contributing cause of the decrease in sediment volume.

CHANGES IN THE DISTRIBUTION OF THE SOLUBLE NITROGEN (J. C. D. WHITE)

It has been shown that the solubility of L and M powders remained almost unaltered even after storage for 700 days at 37° C. H powder, however, although showing little change in solubility when stored at 20° C., rapidly became insoluble when stored at 28·5 and 37° C. whether packed in air or nitrogen. As the loss of solubility of a milk powder is largely due to a decrease in the solubility of the milk proteins, it was decided to determine the distribution of the *soluble* nitrogen and thus indirectly find which fractions of the protein were becoming insoluble and also if soluble nitrogenous decomposition products were being formed. Such an examination would indicate whether the deterioration involved only the casein or whether lactalbumin and lactoglobulin were also involved.

Examination of the fresh powders

For the determination of the initial nitrogen partitions, the fresh powders were reconstituted with water at 20° C. in the manner prescribed for solubility estimation (7) and the 'milk' made up to a known volume. The various protein fractions were separated and estimated by the methods developed by Rowland (11, 12) for liquid milk except that a micro-Kjeldahl procedure (13, 14) was used where possible. The following direct estimations were made: (1) total nitrogen, (2) non-casein nitrogen, (3) non-protein nitrogen, and (4) proteose-peptone plus non-protein nitrogen. From the results so obtained, the following were calculated: casein nitrogen (1)–(2), lactalbumin plus lactoglobulin nitrogen (2)–(4), and proteose-peptone nitrogen (4)–(3).

The proteose-peptone plus non-protein nitrogen was measured by heating the 'milk' for 15 min. at 95° C. to denature the lactalbumin and lactoglobulin. The pH of the 'milk' was then adjusted to 4·6–4·7 (as in (2) for the precipitation of casein alone) to precipitate the denatured 'heat-coagulable' proteins with the casein. The filtrate thus contained only proteose-peptones and non-protein nitrogen. Direct estimations of lactoglobulin were also made.

The nitrogen partitions for the H, M and L fresh powders are given in Table 6. Rowland's (15) average values for the nitrogen partition of fresh milk and Ashworth & Van Orden's (16) data for spray-dried skim milk are included in the table. Comparison of the average values for the three powders with those for fresh milk shows that the percentage of the total nitrogen in the form of lactalbumin plus lactoglobulin in the powders was only 5·5 as compared with 12·5 for fresh milk. This difference was compensated by an apparent increase of 3·7% in casein nitrogen and increases of 2·3 and 0·9% in proteose-peptone

and non-protein nitrogen respectively, i.e. a combined increase of 6·9%. Ashworth & Van Orden's data for other spray-dried milks show a similar but more severe deviation from the nitrogen partition of fresh milk in that almost the whole of the lactalbumin plus lactoglobulin appeared to be denatured, thus causing a corresponding apparent increase in the casein fraction.

Since the milk was dried by the Gray-Jensen spray process, the milk or powder was not subjected to high temperatures during the actual dehydration (17, 18, 19). The deviation of the nitrogen distributions of the fresh powders from that of normal liquid milk would thus depend largely on the temperature [165° F. (74° C.)] and duration (*c.* 30 min.) of the pre-heat treatment, described in detail in Part II.

Rowland (20, 21) has examined the effect of various 'temperature-time' treatments on the nitrogen distribution of milk. He states that lactalbumin and lactoglobulin are rapidly denatured at temperatures of 75° C. and above, and that there is no change in non-protein nitrogen content on heating at temperatures up to 100° C. On continued

Table 6. *Distribution of nitrogen in the fresh powders used in the present work as compared with that found by others for liquid milk and spray-dried skim milk*

	mg. N per g. milk solids					Percentages of total nitrogen					
	Powder				Av. of 32 samples of spray-dried skim milk*	Powder				Av. of 32 samples of spray-dried skim milk*	Liquid milk†
Constituent	H	M	L	Av.		H	M	L	Av.		
Total N	54·1	53·8	55·2	54·5	58·13	100·0	100·0	100·0	100·0	100·0	100·0
Casein N	44·1	44·1	46·0	44·7	52·80	81·5	81·9	83·3	82·2	90·75	78·5
Non-casein N	10·0	9·7	9·2	9·6	5·50	18·5	18·1	16·7	17·8	9·25	21·5
Lactalbumin + lactoglobulin N	3·4	3·3	2·3	3·0	0·23	6·2	6·2	4·1	5·5	0·40	12·5
Proteose-peptone N	3·4	3·1	3·9	3·5	2·30	6·2	5·8	7·0	6·3	3·95	4·0
Non-protein N	3·3	3·3	3·1	3·2	3·06	6·1	6·1	5·6	5·9	5·26	5·0

* Ashworth & Van Orden's (16) mean values for thirty-two samples of spray-dried skim milk.

† Rowland's (15) average values for liquid milk.

heating (30 min.) at 95 and 100° C., very small amounts of proteose-peptone substances are produced. Menefee, Overman & Tracy (22) found that in the preparation of evaporated and condensed milk, pasteurization at 145° F. (62·8° C.) for 30 min. produced no significant differences in nitrogen distribution. On the other hand, fore-warming to 150° F. (65·6° C.) caused slight 'coagulation' of lactalbumin, whereas pasteurization at 190° F. (87·8° C.) for 30 min. and fore-warming to 203° F. (95° C.) both caused complete 'coagulation' of lactalbumin and probably of some lactoglobulin.

These investigations support the view that the changes in the nitrogen distribution of the fresh powders were largely caused by the pre-heat treatment of the liquid milk.

Examination of the stored powders

Using the analytical procedures mentioned above, the nitrogen distribution of H powder was examined at intervals during storage at 37 and 28·5° C. In these analyses, the reconstituted milk was centrifuged to remove the insoluble protein. From the known moisture content of the powder and the percentage solubility of the milk solids, it was possible to calculate the weight of supernatant liquid after centrifuging and thus express

the results as mg. of soluble nitrogen per g. of dry milk solids. Figs. 11 and 12 illustrate the changes in the distribution of the soluble nitrogen during storage of H powder at 37 and 28·5° C. These changes may be summarized as follows:

(1) The same changes took place at both storage temperatures although at a much slower rate at 28·5° C. As would be expected, they ran concurrently with the decrease in solubility of the powder.

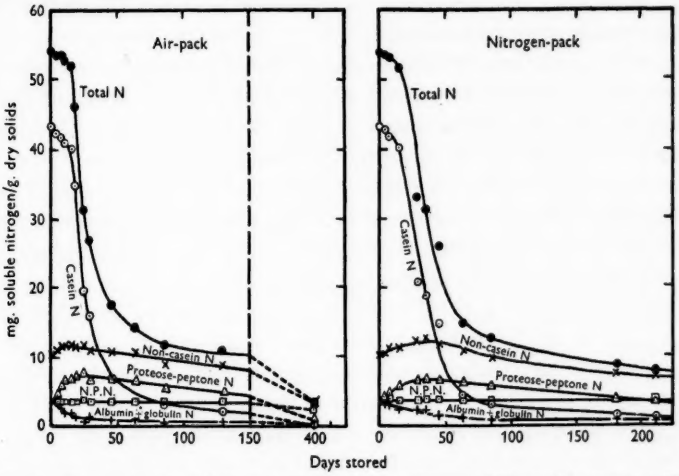

Fig. 11. Changes in the distribution of the soluble nitrogen of H powder stored at 37° C.

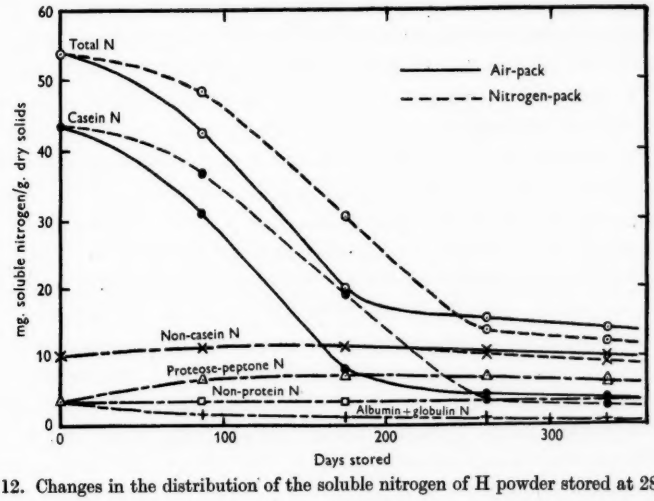

Fig. 12. Changes in the distribution of the soluble nitrogen of H powder stored at 28·5° C.

(2) As storage progressed, the casein rapidly became insoluble and was completely insoluble after 400 days at 37° C. Since the casein nitrogen constituted about 81 % of the total nitrogen, the decrease in soluble casein was closely paralleled by a decrease in total soluble nitrogen.

(3) The proteose-peptone fraction increased to about twice its initial value after about 24 days at 37° C. and also after about 90 days at 28·5° C. and then decreased slowly until

after 400 days at 37° C. only about one-quarter of the initial amount remained in solution.

(4) The level of non-protein nitrogen remained constant showing that there was no severe protein decomposition.

(5) The lactalbumin plus lactoglobulin slowly decreased in solubility and eventually became entirely insoluble (see data for H powder stored for 400 days at 37° C., Fig. 11).

(6) There was a small initial increase in non-casein nitrogen. Thereafter the amount of non-casein nitrogen decreased slowly.

The interpretation of these results is complicated by the fact that the method of nitrogen fractionation, which was developed in the first place for liquid milk, became less efficient when applied to a deteriorated powder. The lactalbumin plus lactoglobulin nitrogen results as determined for H air-packed powder would indicate that these combined fractions decreased from an initial value of 3·3 to 0·0 mg. soluble nitrogen/g. dry milk solids after 400 days' storage at 37° C. These figures were obtained by subtracting the proteose-peptone plus non-protein nitrogen from the non-casein values (cf. p. 313). However, direct estimation of the soluble lactoglobulin nitrogen showed that this fraction remained fairly constant at a value of about 2 mg. nitrogen/g. dry milk solids up to 129 days' storage at 37° C. and then decreased to 0·7 mg./g. solids after 400 days' storage. Ashworth & Van Orden (16) and Menefee *et al.* (22) are of the opinion that the method of estimating lactoglobulin (saturation of a solution containing proteose-peptones, lactalbumin, lactoglobulin and non-protein nitrogen with magnesium sulphate) is not accurate enough, since they found small variable amounts of lactoglobulin in samples of heated milk and milk powders which apparently contained no heat-coagulable protein. Despite this possible inaccuracy, the *estimated* lactoglobulin nitrogen remained at a fairly constant level simultaneously with a redistribution of the other nitrogen fractions and it seems reasonable to conclude that the same uncontaminated lactoglobulin fraction was being estimated each time. If this supposition is correct, then the *calculated* lactalbumin plus lactoglobulin results are too low, since, after 10 days' storage, the lactalbumin values for H air-packed powder at 37° C., obtained by difference, become negative. If, then, the lactoglobulin analyses are assumed correct, the most likely error in the fractionation is that the values for the proteose-peptone plus non-protein nitrogen are too high. A possible explanation may be that lactalbumin and lactoglobulin lose their property of being coagulated by heat and these fractions would then be included with the proteose-peptone and non-protein nitrogen (cf. p. 313). The anomaly of 'negative' lactalbumin values was found also in the nitrogen-packed series and the more deteriorated the powder, the greater was the discrepancy in the analytical results. A similar but even greater discrepancy was found when the fractionation procedure was applied to evaporated milk which had been stored for 10–11 years. Further work is being carried out to find why the separation fails with 'deteriorated' milk protein.

Because of these discrepancies, it is impossible to state quantitatively the changes in solubility of the lactalbumin and lactoglobulin fractions. However, it is probable that most of the lactalbumin of H powder was rendered insoluble after 10 days' storage at 37° C. for the air-pack, and after 28 days for the nitrogen-pack. The direct lactoglobulin estimations suggest that this protein remained soluble until extreme deterioration of the powder was reached after 400 days' storage at 37° C.

The small initial increase in non-casein nitrogen seems real since it was detected in air-

and nitrogen-packed powders both at 28·5 and 37° C. Consequently, at least part of the increase of the proteose-peptone fraction must have been due to casein decomposition. The analytical data do not indicate whether the lactalbumin and lactoglobulin fractions were also involved in the formation of proteose-peptones.

Lampitt & Bushill (23) have also investigated the distribution of the different protein constituents between the soluble and insoluble fractions of milk powders of high moisture contents, stored at 30° C. They state that, contrary to the findings of Supplee & Bellis (24), a proportion of the casein remained soluble after storage, although in one instance the casein decreased from 83·1 % of the total soluble protein to only 2·4 %. It has been shown in the present work that the casein of a milk powder with a high moisture content can become completely insoluble after prolonged storage at a high temperature. Moreover, Lampitt & Bushill (23) say that a relatively large proportion of the lactalbumin and lactoglobulin fractions remained soluble. However, the technique of their analyses did not differentiate between proteose-peptones and the 'heat-coagulable' proteins, and thus soluble proteose-peptones would be estimated as lactalbumin plus lactoglobulin.

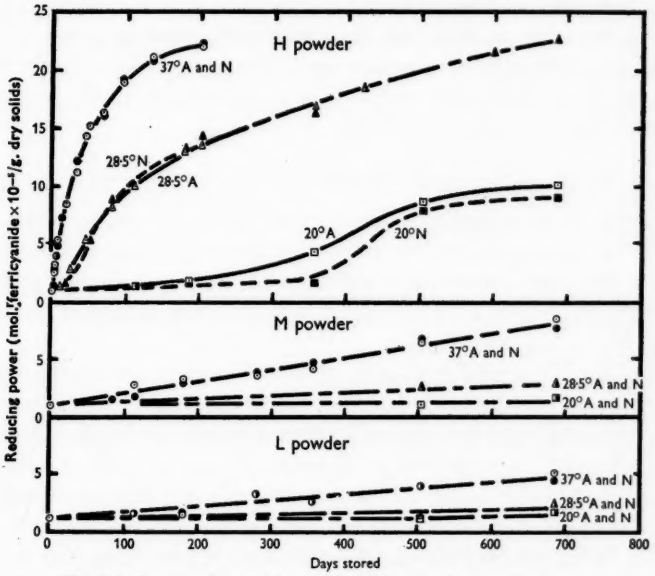

Fig. 13. Increase in reducing power of the powders on storage.

REDUCING POWER OF THE MILK TOWARDS POTASSIUM FERRICYANIDE (C. H. LEA)

Determinations of the reducing power of the fresh and stored milk powders were carried out according to a modification of the method of Chapman & McFarlane (25), which is discussed more fully elsewhere (26). This method depends on the reduction of potassium ferricyanide solution by heating with the powder at 70° C. and pH 5·0, after which the protein is precipitated by trichloroacetic acid and the ferrocyanide produced estimated colorimetrically after development with ferric chloride. Since the reaction now appears to be due to the formation and degradation of a protein-sugar complex, rather than to a simple denaturation of protein with consequent exposure of reducing groups as originally believed, the values obtained directly are expressed as mol. $\times 10^{-5}$ ferricyanide

reduced per g. dry milk solids, rather than in terms of cysteine or glutathione as in the original method.

The results, which are summarized in Fig. 13, stress once more the deleterious effect of a high moisture content in the powder particularly when combined with a high storage temperature. An induction period of the order of 2, 20 and 300 days, with the powder of highest moisture content stored at 37, 28·5 and 20° C. respectively is probably attributable, in part at least, to delayed crystallization of the lactose (cf. Table 3), as probably also is the comparatively slight difference between powders stored in air and nitrogen at the two latter temperatures. No appreciable air-nitrogen difference was detectable at 37° C. The reducing power of M and L powders increased but slowly, and in a linear manner, on storage, no appreciable difference being detectable between the air- and nitrogen-packs.

The effects of moisture content and temperature of storage on the development of reducing power in separated milk powder are thus, on the whole, rather similar to the effects of these factors on a number of the other physical and chemical criteria studied, and have not been tabulated separately.

It will be shown later (p. 334) that the ferricyanide-reducing power of the powder, which can easily be determined as a routine procedure, appears to be closely related to the amino-sugar reaction, which itself seems to be a major factor in the causation of non-fatty deterioration in a variety of foods (27).

Changes in pH, in base-binding capacity and in formol titration (C. H. Lea)

The discoloration of evaporated milk during sterilization has been considered by some workers to be due, in part at least, to interaction between lactose and protein, the aldehyde group of the sugar becoming attached to the protein molecule, possibly at free amino-groups (28, 29). Others have attributed it to a protein-catalysed caramelization of the lactose (30, 31).

As a result of the blocking of free amino-groups by such a reaction the pH of the milk or milk protein might be expected to fall, and its base-binding power to increase, while the content of free amino-groups as measured by formol titration or by the Van Slyke reaction with nitrous acid might be expected to decrease. In fact, the pH of milk is known to decrease and the titratable acidity to increase when liquid milk is strongly heated (32). Recent work, however, has shown that considerable quantities of lactic and of volatile acids including formic are produced, presumably from lactose, when milk is heated under sterilizing conditions (33), and an increase in the acidity of milk on heating cannot therefore be taken as evidence of a reaction involving the free amino-groups of protein. Furthermore, the caramelization of pure sugar is known to produce a fall in pH.

Kass & Palmer (34) have already attempted to use the formol titration method, with or without oxalate (stated by Pyne (35) to eliminate interference by phosphate) as an index of protein-lactose combination in liquid milk during sterilization, but with little success and, on the basis of their results, question the validity of the formol titration of milk as a means of seeking evidence for an aldose-casein condensation. Gould & Frantz (32), in a paper which appeared while the work here reported was in progress, observed *increases* in formol titration when milk was heated at high temperatures, but the change was slight and did not appear to be closely related to browning of the milk. Since the addition of oxalate greatly reduced the increase in formol titration it was concluded that the salts

of the milk were responsible for part of the observed effect. In a still more recent paper Gould, Weaver & Frantz (36) find that the formol titration of evaporated milk is essentially unaffected by storage.

In the present work an attempt has been made to follow interaction between protein and sugar in the experimental dried milks during storage, from the protein side by estimation of free amino-groups by formol titration and by the Van Slyke method, and from the sugar side by estimation of the quantity of sugar combined with the protein.

Method

For investigation of changes in pH, in base-binding power and in formol titration, the stored milk powders were reconstituted with water at 20 or 60° C. to 9·2% solids content, and pH measured by means of the glass electrode. 25 g. of the milk were then brought to pH 8·5 by the stepwise addition, at a standardized rate, of 0·1 N sodium hydroxide, 10 ml. of 25% formaldehyde (previously adjusted to pH 8·5) were added and titration was continued back to, and slightly beyond, pH 8·5. The amount of formaldehyde used was chosen to conform to the recommendations of Levy (37). Corrections for the change in ionic strength resulting from the addition of alkali to the milk and from dilution with the formaldehyde solution were found to be negligible. From the smooth curve obtained, the base-binding capacity of the milk (as milli-equivalents NaOH bound/g. of separated milk solids), and the formol titration (as milli-equivalents NaOH/g. of separated milk solids required to bring the pH back again to 8·5 after the addition of formaldehyde) were calculated. Changes in these values, and in the pH of the reconstituted milk, resulting from the storage of H powder at 37° C. in air or nitrogen for periods up to 203 days are given in Fig. 14.

While titration to an end-point at pH 8·5 is frequently recommended for proteins (38), and a number of proteins certainly appear to give maximum values for the formol titration in this region, e.g. β-lactoglobulin (39), in the case of milk and dialysed milk the displacement of the titration curve produced by formaldehyde seems to be appreciably greater at pH 9·0 than at pH 8·5, as found by Kekwick & Cannan (40) for egg albumin, and several values have therefore been determined also by titrating to this pH. Titration was not continued beyond pH 9·0 and it is not certain that an absolute maximum had been reached. Melnick, Oser & Weiss (41) have recently preferred to terminate the formol titration of enzymic hydrolysates of proteins at pH 9·5.

Kekwick & Cannan (40) have provisionally identified the groups combining with formaldehyde in the formol titration of egg albumin with the ϵ-amino-groups of the lysine residues, and believe that α-amino- and other groups should not interfere to any great extent (cf. also Cannan (38)). When the formol titrations obtained on the fresh, unstored control powders (M and H), in the present experiments are calculated to the lysine content of the dry, separated milk solids, values of 2·69% lysine when measured at pH 8·5, or 2·75% when measured at pH 9·0 are obtained. The amount of lysine found by direct microbiological assay on M control powder was 2·65% (Part V) and 2·9% has been reported in the literature for separated milk powder (42).

A number of the samples of stored powder, after reconstitution at 20 or 60° C., were centrifuged for 15 min. at 3000 r.p.m. and the supernatant liquor separated and titrated as above, to give the 'soluble' formol titration. Data for these samples (Fig. 14) reflected mainly the loss of solubility of the protein on storage, as already shown by

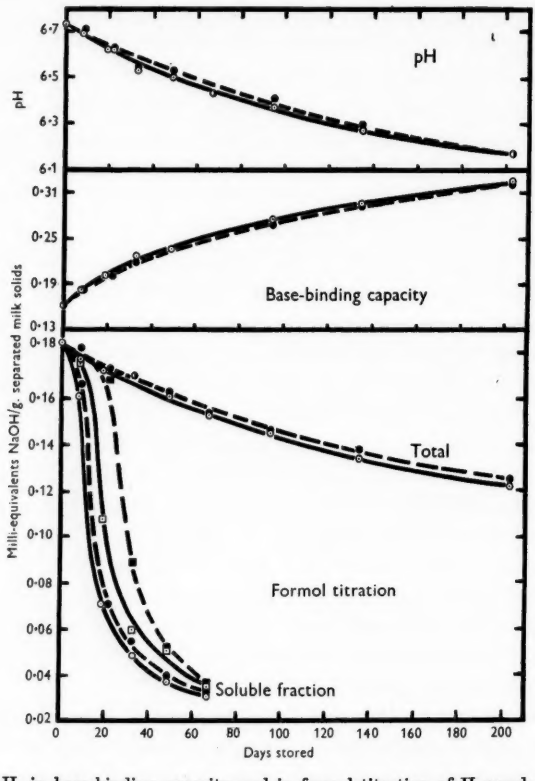

Fig. 14. Changes in pH, in base-binding capacity and in formol titration of H powder on storage at 37° C. Full line = air-stored, broken line = gas-stored samples.

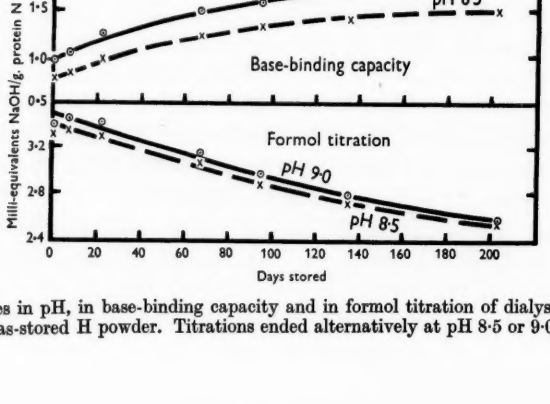

Fig. 15, Changes in pH, in base-binding capacity and in formol titration of dialysed protein from gas-stored H powder. Titrations ended alternatively at pH 8·5 or 9·0.

direct determination (Fig. 9), but served also to show that a very large proportion of the formol titration of the milk was, in fact, due to coagulable protein, or to salts carried down with it.

The progressive fall in formol titration of the high moisture powder on storage at 37° C. was quite definite (Fig. 14), but the change was rather slow, amounting only to about one-third of the initial value after storage for 203 days. Furthermore, the formol titration showed little correlation with loss of solubility, which followed a characteristic S-shaped curve (Fig. 9), or with ferricyanide-reducing power (Fig. 13).

Dialysed milk

An attempt was made to remove anomalies by simplifying the system under investigation, by dialysing several samples of reconstituted, gas-stored, high moisture powders in cellophane at 0° C. prior to potentiometric titration. The materials titrated in this case retained the whole of their protein but only about 37–42% of the original weight of the dry, separated milk solids. A correction was made for the small change in pH produced by the change in ionic strength during the determination, by carrying out a blank titration with KCl in place of NaOH. It is obvious from Fig. 15 that removal of dialysable constituents did not greatly affect the general result. The formol titration of the dialysed control milk as carried out at pH 8·5 and 9·0 could be calculated to a lysine content of the milk protein (assumed to contain 15·7% nitrogen) of 7·8 or 8·0%, or of the dry separated milk solids of 2·6 or 2·7%, values which are again of the expected order of magnitude. However, the fall in formol titration on storage was again almost linear and corresponded to a reduction of only about one-quarter of the initial value after storage at 37° C. for 203 days. The observed increase in base-binding capacity could also be accounted for by the loss of the free amino-groups of 20–25% of the initial content of lysine. The increase in base-binding capacity on storage, when measured after dialysis, was, however, only about one-quarter of that found for the undialysed milk, which indicates that very appreciable quantities of dialysable acids, possibly lactic and formic as produced in heated liquid milk (33), are formed during storage of the high moisture powder at 37° C.

Conclusions

The formol titration of the fresh, unstored separated milk powder whether measured before or after dialysis, was in approximate agreement with the value expected on the assumption that the formol titration is due solely to free amino-groups of the protein, consisting mainly of the ε-amino-groups of the lysine residues.

On storage of separated milk powder of high moisture content at 37° C. for nearly 7 months, a progressive fall in pH, an increase in base-binding capacity and a decrease in the formol titration of the reconstituted material were observed.

The reduction in formol titration during storage, as measured on the undialysed and dialysed milk, was of the order of one-third and one-quarter respectively of the initial value. The increase in base-binding capacity of the dialysed material was also of the order expected, on the assumption that free amino-groups of the protein were being inactivated. The increase in base-binding power of the undialysed milk, however, was considerably larger, indicating the probable production of appreciable quantities of dialysable acid during storage.

The formol titration method, therefore, provides definite evidence of the occurrence

during the storage of milk powders of high moisture content of a reaction involving the free amino-groups of the protein. As will be shown in a later section, however, the results obtained by this method are not in good agreement quantitatively with those obtained by other and perhaps more reliable methods (cf. p. 331).

The protein-sugar reaction (C. H. Lea)

Preparation of the samples for analysis

In order to free the milk from traces of ammonium salts, urea, or other non-protein nitrogenous substances likely to interfere with the Van Slyke determination, and from the large excess of uncombined sugar and from traces of non-sugar reducing substances which would prevent direct estimation of the quantity of sugar combined with the protein, the stored milk powders (5·0 g. dry weight basis) were reconstituted in water at about 20° C., transferred to narrow cellophane sacks and dialysed at 0° C., by a standardized procedure, against frequent changes of distilled water. Under the conditions used, dialysis was virtually complete in less than 3 days, but was normally continued for 5 days, in the presence of a little toluene, to ensure the removal of all free sugar. The dialysed material, which varied from a white, milky fluid without sediment in the case of separated fresh milk or of a control powder, to a suspension of brown, insoluble particles in an almost clear fluid in the case of a badly deteriorated powder, was made up to a total volume of 100 ml., and stored at 0° C. If not used within 2 days the material was discarded and a fresh batch dialysed.

Portions of the well-mixed solution or suspension were weighed out for determination of solids content, and of nitrogen content by the macro-Kjeldahl method. Difficulty in sampling suspensions of the deteriorated products for the estimation of free amino-nitrogen and of combined sugar was overcome by the use of a rapidly emptying, stemless pipette which delivered approximately 4 g. of the well-mixed sample with a reproducibility within the limits of accuracy of the chemical determinations.

Increase in weight of the undialysable fraction

Determination of the solids content of moist materials containing protein and sugar is notoriously a matter of some difficulty as, owing to decomposition, losses recorded during normal high temperature drying procedures tend to be too high.

Two methods of drying were therefore investigated. As a rapid, routine, high temperature procedure, about 20 g. of the dialysed material were dried as rapidly as possible in a large aluminium dish (with cover) on a steam bath, followed by heating for 3 hr. in an air oven at 100° C. Reproducibility was reasonably good and the values obtained were virtually unaffected when drying was prolonged for a further 2–3 hr. In the second method the samples were freeze-dried and subsequently dried over phosphorus pentoxide at 37° C. *in vacuo* to constant weight, a period of approximately 1 week being required. Since the differences between the results obtained by the two methods were comparatively small, the simple, rapid method has been used for routine examination of the stored powders.

The experimental values which have been plotted in Fig. 16 show that, as the result of deterioration during storage, the amount of undialysable material in H powder increased by quantities ranging up to 0·8–0·9 g./g. of non-dialysable ('protein') nitrogen, corresponding to an increase of nearly 5% of the weight of the original dry, separated

milk solids. It will be shown later that this increase in weight of the undialysable fraction of the milk is nearly sufficient to account for the observed loss of Van Slyke amino-nitrogen on the assumption that one molecule of lactose has reacted with each free amino-group destroyed, and is approximately sufficient to account for the combined sugar found by direct estimation (p. 325) and for the observed decrease in total soluble lactose (p. 307).

Degradation of the initial reaction product with formation of dialysable or volatile fragments (e.g. formic and lactic acids, carbon dioxide, water, etc.) would reduce the observed gain in weight, while reaction of any monosaccharide which might be present in very small quantity together with the lactose (cf. p. 331) would also reduce the expected increase in weight.

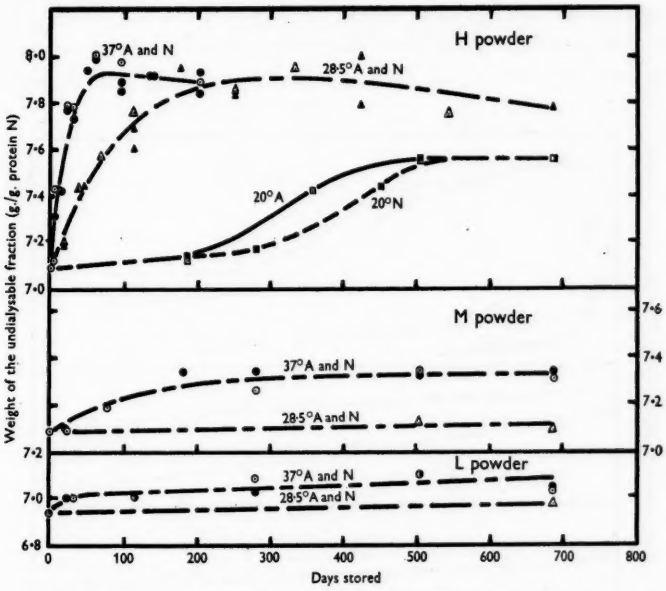

Fig. 16. Increase in weight of the undialysable fraction of the milk on storage.

Nitrogen content of the undialysable fraction

Analysis of the undialysable fraction of the milks for nitrogen content showed that no decrease in undialysable nitrogen occurred on storage, any slight tendency being rather in the direction of an increase. Long-stored, insoluble samples contained 93·5–95·5% of their total nitrogen in an undialysable form. Soluble samples showed an undialysable nitrogen content which varied erratically between 92·5 and 95%, the range of values recorded probably being due to varying permeability or to occasional microscopic leaks in the cellophane sacks used for dialysis. Calculation of all results to the undialysable ('protein') nitrogen basis largely eliminated any small errors which might have arisen from this cause, and also facilitated rejection of the occasional grossly faulty sack. The observed contents of undialysable nitrogen are in agreement with the protein nitrogen values of 94·1 (cf. Table 6), and 94·5% (43) which have been obtained by precipitation methods.

Estimation of free amino-nitrogen by the Van Slyke method

For determination of the free amino-nitrogen content of the stored powders a 4 ml. aliquot of the dialysed, reconstituted sample, prepared as described above (p. 322), was transferred to the reaction chamber of the manometric Van Slyke apparatus and allowed to react with the nitrous acid reagent for 30 min. at a temperature of 20° C. in the presence of capryl alcohol as defoaming agent. An exposure of 30 min. at 20° C. is not sufficient to complete the reaction and the 30 min. values have therefore been corrected by the addition of 2·0 units. Experiments on the application of the Van Slyke method to determination of the free amino-nitrogen content of casein and of fresh and deteriorated milk protein which form the basis for the above correction are described elsewhere (44). Since the correction is applied equally to all values, estimation of the *loss* of free amino-nitrogen which occurs during storage is the same whether the 30 min. or the corrected values be used. All data are reported as mg. free amino-nitrogen per g. undialysable ('protein') nitrogen.

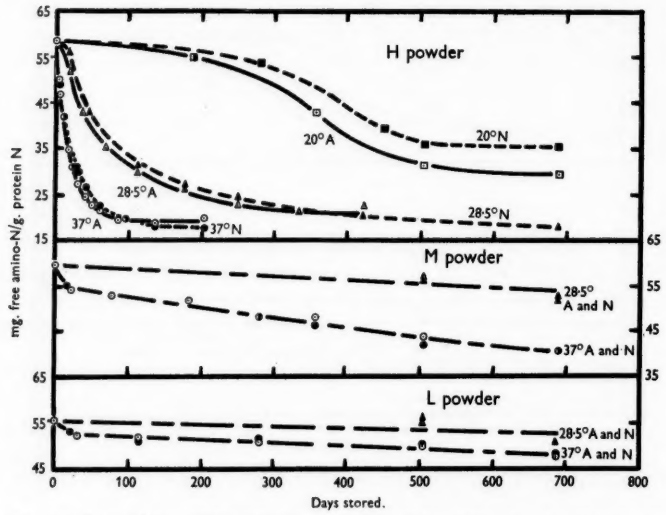

Fig. 17. Loss of free amino-nitrogen by the protein of the stored powders, as determined by the Van Slyke method.

Results

The results which are summarized in Fig. 17 and Table 7 show very marked losses in the free amino-groups of the protein during the storage of milk powder, the change once again being very greatly accelerated by a high moisture content in the powder, particularly when the storage temperature is also high. Evidence of an acceleration produced by the increase in equilibrium relative humidity resulting from crystallization of the lactose is again present in the data for H powder. Slight differences apparent between the rates of deterioration of H powder packed in air and in nitrogen are not greater than can be accounted for by the influence of the atmosphere on the crystallization of lactose at the several storage temperatures (p. 305), and no such effect is noticeable with M and L powders in which the lactose does not crystallize. The reaction responsible for disappearance of the free amino-nitrogen therefore does not require the presence of atmospheric

oxygen. The fact that the reaction comes to a stop, or at least slows down to a very low rate, when only about 70% of the original content of amino-nitrogen has been destroyed will be considered, in conjunction with other relevant data in the general discussion (p. 331).

A point of interest in connexion with the data for M and L powders at 37° C. is the rapid initial loss of 3–6 units of amino-nitrogen which appears to take place early in storage, a change which has no parallel in most of the other criteria determined, but is detectable also in the combined sugar content of the protein (Fig. 18). This point also will be referred to again in the discussion (p. 331).

Table 7. *Loss of free amino-nitrogen (Van Slyke) on storage of skim-milk powder**

		Time taken to lose the stated percentage of initial free amino-nitrogen content (days)							
		Air-pack				Nitrogen-pack			
Powder	Storage temp. (° C.)	5%	10%	20%	50%	5%	10%	20%	50%
H	37	2	3	5	25	2	3	6	30
	28·5	13	17	25	120	17	21	31	140
	20	130	230	320	650	130	300	380	—
M	37	10	40	320	—	10	40	320	—
	28·5	500	—	—	—	500	—	—	—
	20	—	—	—	—	—	—	—	—
L	37	25	—	—	—	25	—	—	—
	28·5	—	—	—	—	—	—	—	—

* Absence of a figure in the table signifies a period of over 700 days.

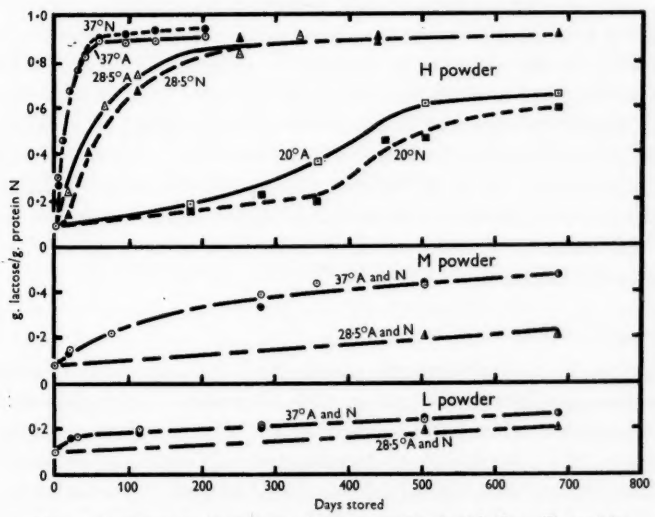

Fig. 18. Increase in the amount of sugar combined with the milk protein, as determined by the direct method.

Estimation of combined sugar

The increase in weight without a corresponding increase in the nitrogen content of the undialysable fraction of the milk, and the decrease in the free amino-content of the protein which accompanied deterioration of the powder on storage suggested that addition or

condensation of a sugar molecule or molecules was occurring at the free amino-groups of the protein. Since reaction with protein in such a way was likely to account for only a small proportion of the very large amount of carbohydrate present, some of which might in any case be destroyed by other reactions not involving protein, it was decided to attempt to follow the course of the protein-sugar reaction by direct estimation of the combined sugar rather than of the residual free sugar. Preliminary attempts to apply a colorimetric orcinol method were disappointing, and attention was directed towards the copper reagent recently described by Somogyi (45).

While any method depending on measurement of reducing power is open to some criticism on the grounds of lack of specificity, the copper reagent is known to oxidize sugars fairly selectively, and no great variety of interfering substances was likely to be encountered in dialysed separated milk. No information was available as to whether it would be possible to recover sugar quantitatively from combination with protein: the browning which accompanied serious deterioration suggested that some at least of the sugar was undergoing decomposition. It was decided therefore first to attempt estimation of the combined sugar *in situ*, without separation from the protein. Subsequent experiments were to be directed towards an investigation of the stability of the protein-sugar link, and examination of the fragments split off by hydrolysis.

Direct determination

The method of Somogyi involves heating 5 ml. of solution containing not more than 3 mg. of glucose or other sugar of equivalent reducing power with 5 ml. of the copper reagent in a freely boiling water-bath until reduction is complete. Lactose reacts comparatively slowly and a period of 30 min. was found preferable to the 20 min. specified by Somogyi for maltose. Glucose is completely oxidized in 10 min. After cooling, potassium iodide is added and the mixture is acidified and titrated with 0·005 N sodium thiosulphate. Determinations and blanks are run in triplicate. The calibration curve prepared from pure α-lactose hydrate is a straight line which very nearly passes through the origin. The presence of protein rendered observation of the end-point more difficult and appeared to cause slightly low recoveries of added sugar, but at the concentration required for the titration of deteriorated samples containing a reasonable amount of sugar such error was negligible.

Determination after acid hydrolysis

The Somogyi reagent is fairly strongly alkaline (pH 10), sufficiently so to be capable of causing some decomposition of the protein with liberation of ammonia and of inorganic sulphur and phosphorus when heated with it at 100° C. Combined sugar may therefore be split off during the determination. In case it should not and the result be too low duplicate determinations were carried out on several samples of powder after the dialysed milk suspension had been heated with N-HCl at 90° C. for 90 min. (or, alternatively, with 2 N-HCl at 100° C. for 60 min.), cooled and neutralized to pH 7. A calibration curve was prepared from pure α-lactose hydrate treated in the same way or, alternatively, from an equimolecular mixture of pure glucose and pure galactose. The presence of sodium chloride reduced the titre of sugar solutions, but the error was not serious at low salt concentrations and was corrected for by calibrating in the presence of suitable concentrations of sodium chloride. Within the limits of accuracy of the sugar deter-

minations the direct and the acid hydrolysis method both gave substantially the same result on the dialysed deteriorated milk powders, which suggests that the combined sugar was, in fact, mainly lactose, and that the reducing power of the sugar towards the alkaline copper reagent was not greatly affected by its union with protein.

Test of the validity of the method

To obtain more precise information as to the behaviour of combined sugar towards the Somogyi reagent protein-glucose and protein-lactose mixtures of known composition were stored at 37° C. and 55% relative humidity for periods up to several months. The results, which are reported elsewhere (8), show that although the estimation of combined sugar by the direct method in the presence of protein gives results which tend to be too low, the error should not be greater than about 10% when the concentration of sugar in the sample is reasonably high.

Results

Values obtained by the direct method on various of the stored milk powders are plotted in Fig. 18. The increase in combined sugar shows obvious signs of relationship with the loss of free amino-nitrogen (Fig. 17), and with the increase in weight of the undialysable fraction of the milk (Fig. 16). The quantitative aspect of this relationship will be considered later (p. 330).

The apparent sugar content of the protein from control powders and from fresh milk, and the possibility of the protein-sugar reaction commencing during processing

The undialysable fraction from fresh, control milk powders showed a small reducing power towards the Somogyi reagent, which could not be eliminated by any reasonable period of dialysis. Even the protein from fresh milk showed a definite, though apparently rather smaller, reducing power. Pending further investigations, it is not clear to what this initial reducing power of fresh milk protein is due, nor is information yet available as to how far the protein-sugar reaction is able to proceed during the normal commercial processes of precondensation, spray- and roller-drying, sterilization, etc.

SUBSIDIARY STORAGE EXPERIMENT (C. H. LEA)

In a previous section (p. 305) it has been shown that while the storage of L and M powders in sealed containers resulted in the maintenance of a reasonably uniform activity of water throughout the experiment, the crystallization of lactose in H powder caused an increase in the activity of water from 41–43% to about 55% relative humidity, a change which began after a noticeable induction period and only reached completion some 3–4, 30–40 and 300–400 days after the commencement of storage at 37, 28·5 and 20° C. respectively. Chemical changes in the powder of highest moisture content did not, therefore, take place at a uniform activity of water.

In order to compare the rates of deterioration at activities of water corresponding to the condition of M powder, which does not crystallize on storage, and of H powder in a sealed container before and after crystallization of the lactose, small samples of M powder were stored in a thin layer at 37° C. over solutions of sulphuric acid giving relative humidities of 29, 43 and 55%. Deterioration was followed by estimation of free amino-nitrogen by the Van Slyke technique, carried out in this case on the powder itself without

dialysis. The results, expressed as free amino-nitrogen on the total nitrogen basis, have been plotted in Fig. 19 as percentages of the initial value.

It is apparent from these data that the initial combination of protein and sugar would have proceeded fairly rapidly in the high moisture powder at 37° C. even if the lactose had failed to crystallize. At 20° C., on the other hand, the difference between the rates of deterioration of H powder before and after crystallization of the lactose was obviously greater, and the effect of crystallization can be traced in the record of most of the criteria of quality measured during the main storage experiment (cf. Figs. 1, 7, 8, 13, 16–18).

The rapid loss of about 10% of the initial content of free amino-groups during the first 20 days of storage at 29% R.H. confirms the similar observation already made on M powder at 37° C. in the main storage experiment (cf. Fig. 17, p. 325).

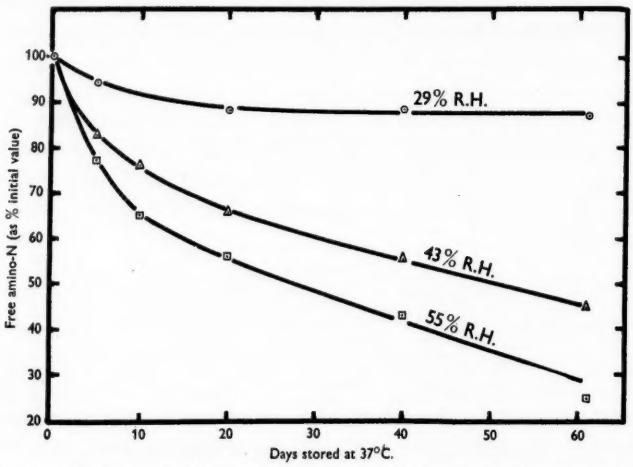

Fig. 19. Loss of free amino-nitrogen by separated milk powder during storage at 37° C. in atmospheres of constant relative humidity.

Bacteriological examination of the stored powders (J. C. D. White)

It is generally recognized and has been shown by Higginbottom (46) that the plate counts of both roller- and spray-dried milk powders tend to decrease during storage. Many samples, however, show little or no change in bacterial count after 6 months or longer. As the present experiment made available three skim-milk powders of different moisture contents which had been stored in air and nitrogen at three temperatures, the opportunity was taken to make a bacteriological examination of the powders after a long period of storage. Of special interest was the possible influence of the different equilibrium relative humidities on the survival of the bacteria. As shown on p. 304, the L and M powders remained at their initial equilibrium relative humidities of 17·5 and 29% respectively throughout storage. On the other hand, the equilibrium R.H. of H powder rose from its initial value of *c.* 42 to *c.* 55% after varying periods which depended on the storage temperature and atmosphere (cf. Fig. 5).

No thorough investigation was attempted of any changes in the bacterial count of the powders after varying lengths of time but the arbitrary period of 600 days' storage was chosen. The H powder stored at 28·5 and 37° C. was not examined since the complete

insolubility of the protein would make a plate count difficult and any result obtained of doubtful value. The plate counts were made by Dr C. Higginbottom using the same technique as for the fresh powders (cf. Part II, p. 295). The results are recorded in Table 8.

Table 8. *The bacterial count of the powders after storage for* 600 *days* (*the counts of the fresh powders are included for comparison*)

Powder	Storage temp. (° C.)	Pack	Plate count/g. powder	
			3 days at 37° C.	5 days at 30° C.
H	20	Air	104,000	224,000
		Nitrogen	264,000	—
Fresh	—	—	132,500	239,500
M	37	Air	171,000	75,000
		Nitrogen	89,000	98,000
	28·5	Air	94,000	79,000
		Nitrogen	148,000	172,000
	20	Air	122,000	151,000
		Nitrogen	232,000	244,000
Fresh	—	—	180,000	281,000
L	37	Air	179,000	187,000
		Nitrogen	246,000	249,000
	28·5	Air	232,500	246,000
		Nitrogen	274,000	249,000
	20	Air	255,000	238,000
		Nitrogen	228,500	256,500
Fresh	—	—	186,000	290,000

Although no definite conclusions can be drawn from the limited data available, the counts indicate a *tendency* to a higher survival rate with decreasing humidity, in nitrogen compared with air, and with decreasing storage temperature. Similar tendencies have been shown by Nichols (47) in respect of storage temperature and nitrogen-packing. Since the apparent increases in some of the plate counts (3 days at 37° C.) are within the experimental error, it seems that even the abnormally high equilibrium R.H. of H powder was insufficient to permit bacterial multiplication.

DISCUSSION

The protein-sugar reaction

The increase in weight of the undialysable (protein) fraction of the milk, together with the accompanying decrease in its free amino-nitrogen content and increase in its combined sugar suggest that, under suitable conditions, protein and reducing sugar react during the storage of milk powder to produce a non-dialysable substance, presumably an addition or condensation product formed by reaction between free amino-groups of the protein and potential aldehyde groups of the reducing sugar.

The relationship between the observed increase in weight of the undialysable fraction of the milk and the increase in its combined sugar content, as determined by direct estimation, is shown in Fig. 20A. The corresponding relationship between the increase in combined sugar and the decrease in free amino-(Van Slyke)-nitrogen content of the protein is given in Fig. 20B. The correlation coefficients in the two cases are 0·94 and 0·99 respectively. These observations cover a range of reaction velocities of the order of several hundreds to one, although observations are naturally limited to the earlier stages of the reaction for those conditions of moisture content and storage temperature which

permitted only relatively slight deterioration during the 2 years of the experimental period.

The deviation between the regression and the theoretical lines drawn in Fig. 20 B shows that approximately 0·88 molecule of lactose was estimated per free amino-group destroyed, the best individual values (gas-stored samples of H powder after 30–60 days at 37° C., or *c.* 250 days at 28·5° C.) giving a ratio of approximately 0·93. Since the direct method used for the estimation of sugar is liable to give results which are too low in about the

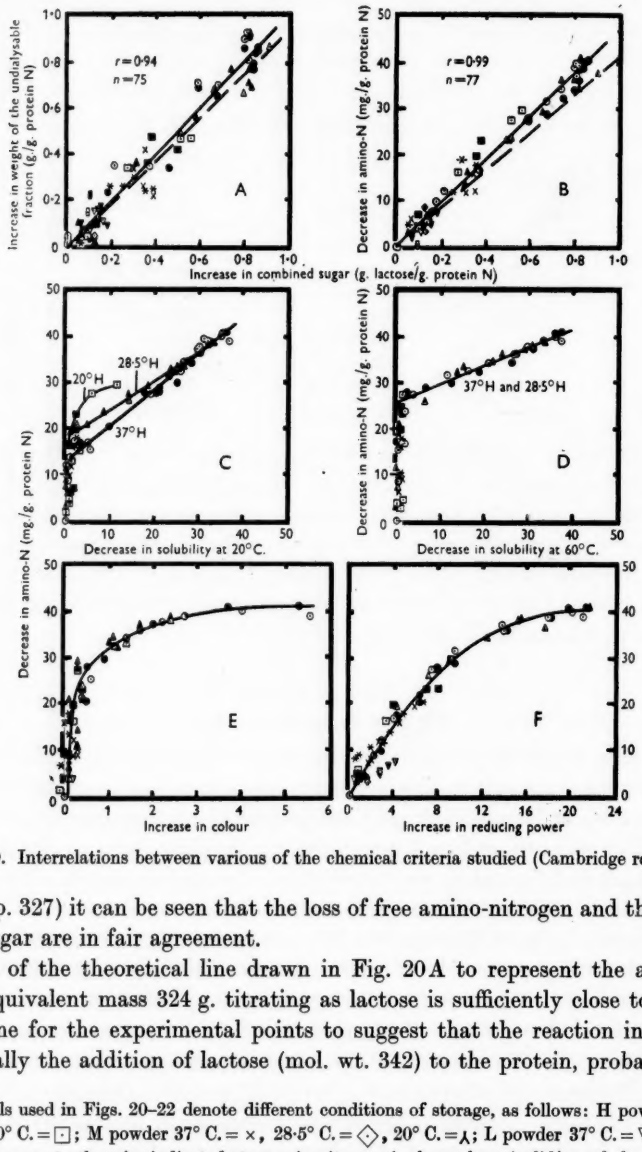

Fig. 20. Interrelations between various of the chemical criteria studied (Cambridge results).*

same ratio (p. 327) it can be seen that the loss of free amino-nitrogen and the increase in combined sugar are in fair agreement.

The slope of the theoretical line drawn in Fig. 20 A to represent the addition of a residue of equivalent mass 324 g. titrating as lactose is sufficiently close to that of the regression line for the experimental points to suggest that the reaction involved is, in fact, essentially the addition of lactose (mol. wt. 342) to the protein, probably with the

* The symbols used in Figs. 20–22 denote different conditions of storage, as follows: H powder 37° C. = ⊙, 28·5° C. = △, 20° C. = ⊡; M powder 37° C. = ×, 28·5° C. = ◇, 20° C. = ⅄; L powder 37° C. = ▽, 28·5° C. = ⊟, 20° C. = ⊡. Where not otherwise indicated storage in nitrogen is shown by a 'solid' symbol.

elimination of a molecule of water (mol. wt. 18). An increase in the sugar values by about 10% would close the gap between the observed and calculated lines.

Despite the poor reproducibility of individual determinations of combined sugar or of increase in undialysable solids the general picture is therefore consistent with the combination of one molecule of lactose with each free amino-group destroyed, part of the combined sugar subsequently undergoing degradation on continued storage, particularly in air.

The relatively rapid drop of 3–6 units in free amino-nitrogen content which occurred early in the storage of M and L powders at 37° C. as well as in the subsidiary storage experiment (p. 328), to be followed by a much slower and linear change, is of interest in that it suggests the presence of a small amount of reactive material which might be either a small proportion of protein amino-groups of greater than average activity or accessibility or a small proportion of a reducing sugar more reactive than lactose. The change is, in fact, of the order of magnitude of that which might be produced by traces of glucose such as have been reported in milk (48, 49), and it is shown elsewhere that glucose, in fact, reacts with protein amino-groups considerably more rapidly than lactose. On the other hand, the reaction of a small amount of glucose appears to be largely suppressed in the presence of a big excess of lactose (8), and variable accessibility of the reacting groups to one another in the solid system is perhaps the more likely explanation.

The sugar-amino-group reaction in H powder at the higher storage temperatures, after a rapid start came virtually to a stop while some 30% of the free amino-nitrogen as determined by the Van Slyke method still remained undestroyed (Fig. 17). While some of the free amino-groups which do not react with reducing sugar may be different, chemically, from those which do (the free α-amino-group of lysine, for example, is known to be much less reactive towards formaldehyde than is the ε-amino-group (50)), it is, nevertheless, true that the ε-amino-groups of the lysine residues probably account for at least 90% of the total free amino-groups present. It appears therefore that some of these lysine amino-groups may be less favourably placed for reaction with sugar molecules, either through being folded within the protein molecule or simply because of the heterogeneity of the solid protein-sugar mass. On the other hand, it is possible that the establishment of an equilibrium between forward and reverse reactions is involved. Other cases in which reactions involving protein amino-groups stop short of completion have been reported. Thus, in the tanning of casein by formaldehyde in the cold under a variety of conditions only about two-thirds of the total Van Slyke free amino-nitrogen is destroyed (51). Similarly, in the production of toxoid from toxin by the action of weak formaldehyde over a long period the reaction ceases when part only of the free amino-nitrogen has been destroyed.

Comparison of the formol and Van Slyke data for free amino-nitrogen

It has already been pointed out that the formol titration of fresh milk powder or of the dialysed milk-protein fraction separated from it, gave a value for free amino-nitrogen in approximate agreement with the lysine content of the milk as found by microbiological assay (Part V), although perhaps very slightly below values which have recently been reported in the literature for milk powder and for casein. Kekwick & Cannan's (40) view that terminal α-amino-groups should not influence the determination to any great extent has also been quoted (p. 319).

The initial Van Slyke values obtained for two samples of fresh milk and for the L, M and H powders corresponded to 10·7, 11·1, 11·1, 11·9 and 11·7% of lysine in the milk

protein, calculated on the nitrogen basis: M and H powders were prepared from the same batch of milk. Milk protein, though obviously variable in composition, usually contains an appreciably higher proportion of lysine than its major constituent casein, for which the best value is probably about 10·1% on the nitrogen or 8·3% on the weight basis (52). The difference of 0·6–1·8% must therefore cover the higher lysine content of the non-casein proteins,* as well as any terminal free α-amino-groups present. Obviously, if the Van Slyke method is yielding results on the fresh protein which are too high, the error cannot be very great.

Both formol and Van Slyke methods therefore yield reasonable initial values on the fresh-milk powders. The Van Slyke method, supported by the dry weight of the undialysable fraction and by the combined sugar determinations, however, indicates a much greater loss of free amino-groups (and hence of lysine) from the protein during storage than the formol titration method. Moreover, the shape of the curve relating loss of amino-group to time of storage is also different for the two methods, a greater proportion of the total loss occurring during the early part of storage when measured by the Van Slyke method.

Microbiological assay (cf. Part V) on H powder after enzymic hydrolysis indicated a loss of lysine during storage for 3 months at 37° C. of the order of 40%, which is greater than the loss of free amino-groups found by the formol titration (15–20%), but less than that found by the Van Slyke method (*c.* 65%). Microbiological assay after acid hydrolysis showed a smaller loss of lysine of the order of 15%.

It is difficult to imagine that the Van Slyke method should yield results on deteriorated powder which are too low, although it is by no means difficult to visualize partial regeneration of lysine during hydrolysis of the protein, particularly with acid, prior to microbiological assay. The reason for the lack of agreement between the formol and Van Slyke determinations on deteriorated powder, however, is not yet clear. It is not due to regeneration of free amino-groups during the formol titration since titration of deteriorated powder to pH 8·5 produces no increase in Van Slyke nitrogen. One possibility that has not yet been investigated is that the basic character of the amino-group is not sufficiently weakened by the first reaction it undergoes during deterioration of the powder to prevent partial titration under the conditions of the formol estimations. Whatever the reason for the discrepancy there seems little doubt, from the balance of the evidence available, that the Van Slyke method gives the more useful picture of the changes occurring in the free amino-groups of the protein during the storage of milk powder. Similar evidence has also been obtained for an artificial protein-glucose system (8).

The possible nature of the mechanism whereby the interaction of protein amino-groups and reducing sugar apparently leads to the production of undesirable physical and chemical changes in both protein and sugar, and to the loss of biological value of the protein is discussed elsewhere (Part VI, p. 359 (8)).

Solubility

The relationship between the protein-sugar reaction, as measured by the decrease in free amino-(Van Slyke)-nitrogen, and the solubility of the powder, as determined after reconstitution in water at 20 or at 60° C., is shown in Figs. 20C and D. According to both

* Block & Mitchell (53), for example, quote 7·9, 9·6 and 11·4% for the lysine contents (weight basis) of casein, lactalbumin and β-lactoglobulin respectively, although they also give the obsolete value of 7·5% for the total protein.

sets of data a very considerable loss of free amino-nitrogen occurred before the onset of loss of solubility, the figures at 60° C. indicating that as much as two-thirds to three-quarters of the reactive amino-nitrogen, or nearly half of the total amino-nitrogen, was destroyed before solubility was appreciably reduced. Loss of solubility in water at 20° C., which apparently represents an earlier stage in deterioration, commenced at somewhat lower levels of amino-group loss, particularly when the powder had been held at high storage temperatures, but the 'lag' period was still very marked. This delayed onset of deterioration in solubility in relation to loss of free amino-nitrogen has also been demonstrated in experiments with artificial protein-sugar systems (8). It follows from these results that a considerable proportion of the free amino-groups of the protein molecule can combine with reducing sugar without appreciably affecting the solubility of the protein.

The question still remains whether insolubility is finally produced (*a*) simply by combination of still further amino-groups each with a molecule of reducing sugar, or (*b*) is the result of an induced denaturation of the protein molecule, or of degradation of the protein-sugar complex by secondary reactions between the now adjacent protein molecules and carbohydrate chains. The fact that a very small quantity of glucose, able to combine only with about one-eighth of the free amino-groups of the protein can, under suitable conditions, give rise to serious discoloration and insolubility of over two-thirds of the protein, this deterioration occurring after completion of the fall in amino-nitrogen (8), strongly supports hypothesis (*b*), as does evidence derived from the measurement of discoloration, reducing powder, evolution of carbon dioxide, etc., in the main storage experiment. The initiation of loss of solubility in cold water at progressively lower values of amino-nitrogen loss as the temperature of storage of the powder increases (Fig. 20C) is also suggestive of such a mechanism.

It has already been pointed out (p. 313) that the fresh powders differed appreciably from fresh milk in possessing a lower lactalbumin plus lactoglobulin, and a slightly higher apparent casein and proteose-peptone content, these changes presumably being a result of the pre-heating of the liquid milk. As deterioration progressed during storage, most of the casein and probably most of the lactalbumin became insoluble, while the lactoglobulin appeared to remain soluble until an extreme stage of deterioration had been reached. A small amount of proteose-peptone was produced, of which part at least arose from decomposition of the casein, but the absence of any increase in non-protein nitrogen indicated that no drastic fragmentation of the protein molecule had occurred.

Colour

The nature of the relationship between the protein-sugar reaction and discoloration of the powder is shown in Fig. 20E. As with solubility in hot water about three-quarters of the reactive or one-half of the total free amino-nitrogen disappears, presumably as the result of combination with sugar, before any very marked change in colour occurs. Even when the primary sugar-amino-group reaction has almost ceased the development of discoloration proceeds at an almost undiminished rate. Apparently the protein-sugar complex first formed is not coloured, but becomes so on degradation. The experiments in which dialysed milk protein was stored in presence or absence of added reducing sugar have shown that the complex is unstable under conditions which cause no discoloration of protein or sugar alone (8). The possibility that discoloration may be due in part to the caramelization of lactose catalysed by amino-nitrogen is discussed elsewhere (8).

Reducing power

The increase in reducing power of the whole powder towards ferricyanide, as measured by the Chapman & McFarlane (25) method shows a good correlation with the progress of the primary sugar-amino reaction under the various storage conditions (cf. Figs. 13, 16–18) until about three-quarters of the reactive or one-half of the total free amino-nitrogen has reacted with sugar (Fig. 20 F). This behaviour is consistent with the observation that neither protein nor sugar alone develops on storage the power of reducing ferricyanide under conditions which cause the mixture (complex) rapidly to do so (26).

For powders stored at 37 and 28·5° but apparently not at 20° C., the reducing power of the milk continues to increase, after the primary amino-group-sugar reaction has almost ceased, thus suggesting more extensive decomposition of the protein-sugar complex at the higher storage temperatures. The data on colour and weight increase (Figs. 3 and 16) have already indicated more extensive decomposition of the protein-sugar complex under such conditions. It has been shown elsewhere that soluble, dialysable, non-nitrogenous substances, which are presumably degradation products of the protein-sugar complex or caramelized sugar, contribute to the reducing power of powder of high moisture content stored for long periods at 37° C. (26).

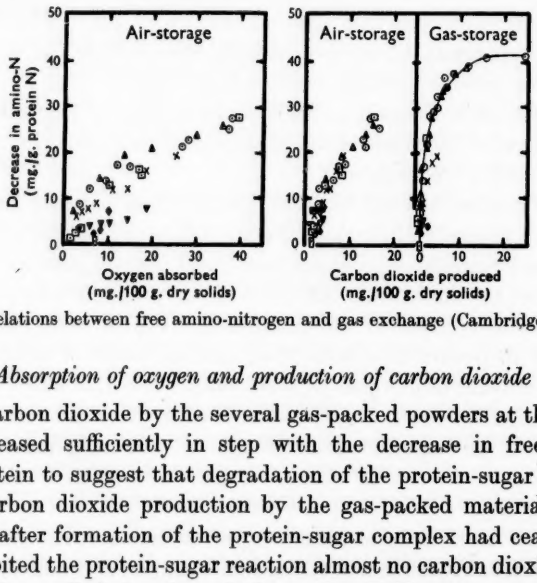

Fig. 21. Relations between free amino-nitrogen and gas exchange (Cambridge results).

Absorption of oxygen and production of carbon dioxide

Production of carbon dioxide by the several gas-packed powders at the several storage temperatures increased sufficiently in step with the decrease in free amino-nitrogen content of the protein to suggest that degradation of the protein-sugar complex was the main source of carbon dioxide production by the gas-packed material (Fig. 21). This process continued after formation of the protein-sugar complex had ceased. Under conditions which inhibited the protein-sugar reaction almost no carbon dioxide was produced by gas-packed, and very little by air-packed, powder (Table 4).

In the air-packed cans the production of carbon dioxide was much more rapid (until all free oxygen had been absorbed after which the rate fell to that of the gas-packed material), but appeared still to be largely derived from the protein-sugar complex (Fig. 21). Absorption of oxygen by the air-packed materials, however, showed a less obvious connexion with loss of amino-nitrogen. The powder of high moisture content absorbed oxygen roughly in proportion to the progress of the amino-group-sugar reaction, indicating that the oxygen was probably being used largely in degradation of the protein-sugar complex. With M powder at 37° C., and particularly with materials such as L

powder at 37° C., or M powder at 28·5° C., in which the protein-sugar reaction was very slow, however, a relatively too rapid absorption of oxygen over the long period of the test suggests the existence of another system slowly absorbing oxygen, with but little production of carbon dioxide. Such a system might perhaps be provided by fat, of which a small proportion was still present in these 'separated' milk powders. This point will be considered again later in connexion with deterioration in flavour.

The amount of complete degradation to carbon dioxide and water which occurred was comparatively small, the maximum amount of carbon dioxide liberated by the air-packed H powder during storage for 203 days at 37° C., for example, corresponding to rather less than 1% of the weight of sugar bound by the protein, or less than 0·05% of the weight of the protein-sugar complex.

Flavour

A notable feature of the taste panel results was the extreme rapidity with which 'off'-flavour developed under the more unfavourable conditions of storage; H powder, for example, passed below the acceptance level after about 2 days in air at 37° C. On the other hand, gas-packed M and L powders at 20° C. were still well above the acceptance line after 2 years (Table 1).

Since the senses of taste and smell are frequently affected by amounts of chemical change which appear small when measured by ordinary chemical means, the rapidity with which serious 'off'-flavours developed in some of the experimental powders does not in itself disprove any connexion between taste and the chemical criteria measured. There is, in fact, when due allowance is made for the wide range of stabilities encountered and for inaccuracies inherent in the tasting technique, a general parallelism between flavour deterioration and a number of the chemical criteria, a parallelism which improves as the range of experimental conditions included in the survey is restricted.

The most obvious discrepancy between the tasting data and chemical tests based directly on the protein-sugar reaction is that of the relative keeping properties of the powders when packed in air and in nitrogen. As already pointed out (p. 324) there is no evidence that the rate of combination of protein amino-groups and reducing sugar is influenced by the atmosphere in the container, except indirectly as a result of changes in the activity of water resulting from the crystallization of lactose. This occurred only in H powder, and the effect was generally quite small, being greatest for storage at 20° C. Chemical tests based on secondary changes involving the protein-sugar reaction, e.g. loss of solubility and discoloration, also seemed to be affected only slightly by packing in nitrogen instead of in air, and here again part at least of the protective effect of gas-packing could be attributed to delayed crystallization of the lactose in H powder.

On the other hand, differences between the rates of deterioration of flavour in air- and inert gas-pack were very marked indeed for all three powders at all three storage temperatures, and M and L powders at least, showed no difference in the rates of combination of protein and sugar in air and in nitrogen to account for the more rapid development of 'off'-flavour in air-storage (Figs. 16–18).

When deterioration in flavour is plotted against decrease in free amino-nitrogen the correlation is found to be quite good for the gas-stored, but less so for the air-stored samples (Fig. 22 A). It appears that, while the protein-sugar reaction is probably fairly directly responsible for the deterioration in flavour of gas-stored powders, in air a

considerable additional development of 'off'-flavour occurs, and is present even under conditions which permit little or no reaction between protein and sugar.

Production of carbon dioxide by the gas-stored samples also shows a good correlation with deterioration in flavour (Fig. 22B), and is presumably a measure of the thermal or spontaneous degradation of the protein-sugar complex (or, possibly, of protein-catalysed caramelization of sugar), which in turn probably accounts for the development of 'off'-flavour and colour in the gas-stored powders. As previously pointed out, 'off'-flavour in gas-stored samples has frequently a predominantly 'caramelized' or 'burnt-sugar' character.

Absorption of oxygen by the air-stored samples, which also shows some parallelism with deterioration in flavour, is probably accounted for partly by oxidation of the decomposing protein-sugar compound with evolution of much carbon dioxide, and partly

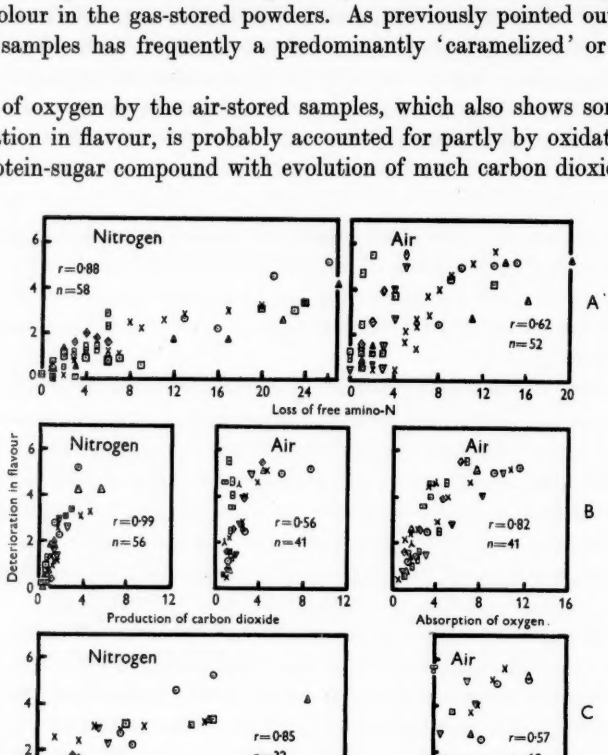

Fig. 22. Relations between flavour and various of the chemical criteria (Cambridge results).

by oxidation of some other component of the system (possibly fat) with evolution of relatively little carbon dioxide. The former mechanism is of major importance for samples of high, and the latter for samples of low, moisture content. The odour and flavour developed by powders of high moisture content deteriorating in air have a pronounced 'stale and glue-like' character.

The reducing power of the powders towards potassium ferricyanide shows a fair degree of correlation with deterioration in flavour (Fig. 22C), but again the relationship between reducing power and flavour varies appreciably with storage conditions, and particularly with the atmosphere in the container: whether the gas in the can be air or nitrogen, reducing power develops at approximately the same rate, while 'off'-flavour appears more rapidly in air.

To summarize, it appears that the failure of the chemical tests to co-ordinate more successfully with deterioration in flavour over a wide range of moisture content of the powder and conditions of storage is probably due to the fact that 'off'-flavour as assessed by the panel is not a simple phenomenon resulting from one reaction, but is a complex built up from a number of components resulting from several types of reaction.

These include (*a*) anaerobic degradation of the protein sugar-complex (and perhaps, the protein-catalysed caramelization of sugar) as found in gas-stored powders of high moisture content, (*b*) oxidation of the protein-sugar complex or of its degradation products (and, perhaps of caramelized sugar), proceeding together with (*a*) in air-stored powders of high moisture content, and (*c*) oxidation of some constituent or constituents of the powder, possibly fat, which proceeds independently of the protein-sugar reaction and is mainly of importance in air-packed powders of low moisture content stored for long periods.

Since different small groups of tasters cannot be expected to attach precisely the same relative significance to 'off'-flavours of several types, some divergence of opinion between panels as to the effects on palatability of the various storage factors studied in the present work is inevitable. Comparison of the results given by the panels operating independently at Cambridge and at the Hannah Institute, however, shows that the effect of such differences of opinion, as well as of an overall difference in severity of marking, has not been sufficient to obscure a substantial measure of agreement on the main points at issue (Table 1).

Effect of moisture content of the powder, of storage temperature and of atmosphere in the container on the rate of deterioration

Consideration of the data accumulated during storage of the experimental powders at three levels of moisture content, at three storage temperatures and in two atmospheres brings out many points of similarity. By all criteria the moisture content of the powder was the factor of greatest importance in determining storage life, the L (3·0%) and M (5·0%) powders being relatively stable, with a definite advantage in favour of the former, while the H (7·6%) powder was very unstable. Part of the large difference in behaviour between H powder and the others can be attributed to the fact that the α-lactose originally present in a 'glassy' supersaturated form crystallized in H powder (the process requiring a few days at 37°, a few weeks at 28·5° and a few months at 20° C.), thereby increasing the activity of the water in the residual protein-sugar mixture and accentuating the difference between the high moisture and the other powders, in which crystallization did not occur even after many months at 37° C.

The temperature coefficient of deterioration of H powder was also very high, the rate usually being increased by a factor of at least 5 for each 8·5° C. increase in temperature, corresponding to a Q_{10} of at least 6. This extreme sensitivity to slight variation in moisture content in the upper part of the range tested, and to slight differences in storage temperature seemed to be a characteristic of the protein-sugar reaction. The effect of change in moisture content was smaller and the temperature coefficients much lower (Q_{10} of the order of 2) when change could be detected, e.g. in flavour or in gas exchange, under conditions in which but little reaction between protein and sugar occurred (e.g. M powder at 28·5 or 20° C., and L powder at 37, 28·5 and 20° C.).

The probable connexion between delayed crystallization in gas-packed as compared

with air-packed H powder and the slightly slower protein-sugar reaction in the former material, particularly at 20° C., has already been pointed out, and a small amount of evidence produced suggesting that some cause other than slight fortuitous differences in moisture content was responsible. On the other hand, such small differences in moisture content were shown to account quite satisfactorily for the considerable 'scatter' in crystallization time among individual cans of air-packed H powder at 20° C. (cf. p. 306), and the question of the cause of the air-gas difference observed with this powder cannot be regarded as satisfactorily settled. The influence of storage atmosphere, however, on all criteria excepting only flavour and gas exchange, for which separate explanations are available, was comparatively small.

Summary

1. Three spray-dried separated milk powders with moisture contents of 2·9, 4·7 and 7·3% (3 hr. air oven) or 3·0, 5·0 and 7·6% (20 hr. vacuum oven) were packed in air and in almost pure nitrogen, in gas-tight cans, and stored at 20·0, 28·5 and 37° C., for a period of nearly 2 years.

2. The powders were examined at intervals for palatability, colour, pH, equilibrium relative humidity (indicating crystallization of the lactose), conversion of β-lactose to α-lactose hydrate, decrease in total soluble lactose, absorption of oxygen, production of carbon dioxide, solubility in water at 20 and at 50 or 60° C., changes in the distribution of soluble nitrogen, reducing power towards potassium ferricyanide, base-binding capacity, formol titration, weight of the undialysable fraction, free amino-nitrogen by the Van Slyke method, sugar attached to the protein, and bacterial content.

3. Little change was observed in the powders of low and medium moisture content except in palatability and gas exchange at the higher temperatures. The powder of the highest moisture content, particularly at the higher storage temperatures, rapidly became unpalatable, discoloured and insoluble. Its pH, free amino-nitrogen and soluble lactose content fell, the amount of sugar attached to the protein and the reducing power of the powder towards potassium ferricyanide increased. Oxygen was absorbed and carbon dioxide produced.

4. It was concluded that a major cause of deterioration in powder of high moisture content, particularly at high storage temperatures, is a reaction involving the free amino-groups of the milk protein, which will consist very largely of the ϵ-amino-groups of the lysine residues. The first stage of this reaction appears to be between the protein amino-groups and the potential aldehyde group of reducing sugar.

5. The rate of the protein-sugar reaction is largely determined by the moisture content or, more correctly, by the activity of moisture in the powder, and is very much greater at 7·6 than at 5·0 or 3·0%. Crystallization of lactose, which occurred only in the powder of highest moisture content, increased the activity of the residual water in the sealed container, and further accelerated deterioration.

6. The temperature coefficient of the formation (and degradation) of the protein-sugar complex is high (at least 6), and moisture contents which can be tolerated under temperate conditions for long periods will be unsatisfactory at high storage temperatures.

7. The reaction between protein and reducing sugar takes place in at least two stages, the primary combination resulting neither in discoloration nor in loss of solubility, which follows only as the result of secondary changes, the nature of which is not fully understood.

8. Those physical and chemical properties of the milk powders which depended essentially on the protein-sugar reaction were influenced only slightly by the nature of the atmosphere in the container.

9. The protein-sugar reaction resulted in the production of 'off'-flavours described as mainly 'heated' or 'caramelized' in the case of gas-packed powders, and as 'stale' and 'gluey' in the case of the air-packed powders. The latter were considered the more objectionable.

10. Evidence was also obtained of an oxidative reaction (or reactions), independent of the protein-sugar change, which produced 'off'-flavour in powders stored for long periods at moisture contents too low for the protein-sugar reaction to occur. It is possible that the small amount of fat present in the powders was involved.

11. These two factors resulted in a decided advantage for gas-packing, in so far as palatability was concerned, at all moisture contents and storage temperatures.

12. Owing to the complex nature of the causes of 'off'-flavour, no single chemical test correlated satisfactorily with the tasting panel score over all conditions of storage, the biggest discrepancy usually being experienced between air-packed samples on the one hand and gas-packed on the other. There was, however, a general parallelism between several of the chemical criteria measured and palatability, which was improved as the range of composition and storage conditions was narrowed.

13. Several of the chemical tests linked up fairly directly with the protein-sugar change, and of these the measurement of reducing power towards ferricyanide by the Chapman & McFarlane technique (25, 26) showed promise.

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PART IV. CHANGES IN THE BIOLOGICAL VALUE OF THE PROTEINS

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(With 1 Figure)

The observations which have led to the inquiry now described are outlined in Part I of this report.

EXPERIMENTAL

General procedure

Animals and technique of tests

In most of the tests described below, the biological value (b.v.) and true digestibility (t.d.) of the proteins of the milk powders were measured on rats by the method of Mitchell (1, 2), and at the same time the protein efficiency (p.e.) (i.e. the gain in weight of the animal expressed in g./g. protein intake) was determined by the method of Osborne, Mendel & Ferry (3). The milk powders were included in the diets to supply approximately 8% protein (N × 6·38). Table 1 gives the composition of the experimental diets, Table 2 their analysis and a description of the milks tested in the various experiments.

In each test carried out by the balance-sheet method (1, 2) twelve female rats were used in litter-mate comparisons, and during the course of any one experiment all rats received each diet in turn. Full details of the experimental technique have been described by Henry, Kon & Watson (4). A maximum of four milk powders was tested simultaneously by this method. Male rats were used in the growth tests (3), also in litter-mate comparisons, each substance tested being offered to six rats. The animals received the experimental diets for 4 days in order that they might become accustomed to the change from the stock diet; subsequently records of weight increases and of diet intake were kept for a period of 4 weeks.

Milk powder used as a standard for comparison

It will be recalled that the H powder was prepared by raising the moisture content of a quantity of M powder whereas the L powder was made from a separate batch of liquid milk (cf. p. 294). Though there is no doubt that originally the three powders did not differ in the nutritive values of their proteins (cf. p. 347), M powder rather than L powder was taken as a standard for comparison; for this purpose cans of it, gas-packed and stored at −20° C. until needed, were used.

The selection of the powders examined in biological tests was guided by the results of chemical measurements (Part III).

Effect of the addition of cystine to samples of dried skim milk, freshly prepared, or stored for 5 years (Exp. 1)

It was originally thought (5) that cystine might be the amino-acid affected in the deterioration, on storage, of dried skim-milk powder. An experiment to test this view was, therefore, the first to be done, while the powders used in the main study were being made ready.

L-Cystine was added to the original deteriorated milk powder (6, 7), which by this time had been stored for 5 years, and to the freshly prepared low moisture powder (L control)

Table 1. *Percentage composition of the experimental diets*

Milk tested*	Diet no.	Milk	Salts†	Margarine fat	Sugar granulated ground	Potato starch	Rice starch
Dried in 1939‡	257	21·7	4·0	10·0	12·0	10·0	42·3
Dried in 1939‡ + 0·18% cystine	258	21·9	4·0	10·0	12·0	10·0	42·1
L	259	22·0	4·0	10·0	12·0	10·0	42·0
L + 0·18% cystine	260	22·3	4·0	10·0	12·0	10·0	41·7
M	263§	23·2	4·0	10·0	12·0	10·0	40·8
H	267‖	23·7	4·0	10·0	12·0	10·0	40·3
Freeze-dried	273	22·6	4·0	10·0	12·0	10·0	41·4
Freeze-dried + 0·18% cystine	274	22·7	4·0	10·0	12·0	10·0	41·3
M + 2·5% lysine	282	22·4	4·0	10·0	12·0	10·0	41·6
H + 2·5% lysine	283	22·6	4·0	10·0	12·0	10·0	41·4
M + 1·25% lysine	284	22·9	4·0	10·0	12·0	10·0	41·1
M + 5% lysine	285	21·6	4·0	10·0	12·0	10·0	42·4
H + 1·25% lysine	286¶	23·4	4·0	10·0	12·0	10·0	40·6
M + 0·5% histidine + 0·5% arginine	290	22·2	4·0	10·0	12·0	10·0	41·8
H + 1·25% lysine + 0·5% histidine	291	23·0	4·0	10·0	12·0	10·0	41·0
H + 1·25% lysine + 0·5% histidine + 0·5% arginine	292	22·6	4·0	10·0	12·0	10·0	41·4

* Except where otherwise stated all samples of skim milk were spray-dried. L = low, M = medium, H = high, content of moisture (cf. Part II).

† de Loureiro (1931). *Arch. Patol., Lisboa*, **3**, 72. ‡ Tested in 1945. § 263 and 263A.

‖ 267, 267A, 267B, 267C, 267D, 267E, 267F and 267G. ¶ 286 and 286A.

Table 2. *Analysis of the diets used in the various experiments*

Diet no.	Description of the dried skim milk tested*	% N	% moisture
	Exp. 1		
257	Spray-dried, prepared in 1939	1·280	9·02
258	Spray-dried, prepared in 1939 + 0·18% cystine	1·280	9·04
259	L control	1·272	7·98
260	L control + 0·18% cystine	1·277	8·18
	Exp. 2		
273	Freeze-dried	1·238	9·02
274	Freeze-dried + 0·18% cystine	1·242	9·15
	Exp. 3		
259	L control	1·269	8·22
263	M control	1·282	8·54
	Exp. 4		
267	H, air-packed, stored at 37° C. for 8 days	1·288	9·46
267A	H, air-packed, stored at 37° C. for 28 days	1·297	9·06
267B	H, gas-packed, stored at 37° C. for 28 days	1·282	9·39
267C	H, air-packed, stored at 37° C. for 85 days	1·273	9·34
	Exp. 5		
263	M control	1·282	8·61
263A	M, air-packed, stored at 37° C. for 182 days	1·277	9·02
267D	H, air-packed, stored at 28·5° C. for 176 days	1·258	9·50
267E	H, gas-packed, stored at 28·5° C. for 176 days	1·260	9·33
	Exp. 6		
263	M control	1·248	9·00
282	M control + 2·5% lysine	1·280	9·06
267F	H, gas-packed, stored at 37° C. for 60 days	1·244	9·41
283	H, gas-packed, stored at 37° C. for 60 days + 2·5% lysine	1·259	9·33
	Exp. 7		
263	M control	1·269	9·87
284	M control + 1·25% lysine	1·272	9·64
282	M control + 2·5% lysine	1·270	9·58
285	M control + 5% lysine	1·267	9·95
267F	H, gas-packed, stored at 37° C. for 60 days	1·267	9·73
286	H, gas-packed, stored at 37° C. for 60 days + 1·25% lysine	1·283	9·20
	Exp. 8		
263	M control	1·267	9·30
290	M control + 0·5% histidine + 0·5% arginine	1·272	9·06
267G	H, air-packed, stored at 37° C. for 60 days	1·256	9·20
286A	H, air-packed, stored at 37° C. for 60 days + 1·25% lysine	1·256	9·63
291	H, air-packed, stored at 37° C. for 60 days + 1·25% lysine + 0·5% histidine	1·265	9·33
292	H, air-packed, stored at 37° C. for 60 days + 1·25 lysine + 0·5% histidine + 0·5% arginine	1·268	9·43

* L = low, M = medium, H = high, content of moisture (cf. Part II).

which was intended for use in the main experiments. On the assumption that all the cystine of the deteriorated dried milk had been destroyed during storage, the amino-acid was added to both powders at the rate of 0·5% of the milk proteins. The b.v. and t.d. of these milks were determined by the balance-sheet method (1, 2), but a test by the rat-growth technique (3) was not included as there was not enough of the deteriorated milk left. The results of the experiment are given in Tables 3 and 5.

It will be seen that a statistically significant improvement in the b.v. from 90·4 to 95·2 was observed when cystine was added to the freshly prepared dried skim milk. On the other hand, no significant improvement resulted from the addition of cystine to the deteriorated sample, b.v.'s of 77·9 and 76·6 respectively being obtained with and without it.

Table 3. *Mean results for groups of* 12 *rats for the biological values and true digestibilities of the proteins* (N × 6·38) *of the milks studied in the various experiments*

				Biological value		True digestibility	
Exp. no.	Age of rats at start (days)	Weight of rats at start (g.)	Diet no.	Numerical result and its S.E. of the mean	Statistical significance*	Numerical result and its S.E. of the mean	Statistical significance*
1	26	56–57	257	76·6±1·79	5% = 2·89 1% = 3·96 0·1% = 5·39	87·3±0·69	5% = 2·61 1% = 3·58 0·1% = 4·87
			258	77·9±1·82		86·1±1·21	
			259	90·4±2·09		92·4±0·75	
			260	95·2±1·63		95·0±0·74	
2	25–26	43–57	273	90·1±2·50	S.E.M. = 2·87 P = 1 : 34	92·5±1·16	S.E.M. = 0·83 P = 1 : 29
			274	97·3±1·04		94·5±0·93	
4	24–25	54–58	267	86·9±1·35	5% = 3·90 1% = 5·35 0·1% = 7·28	90·9±0·57	5% = 2·46 1% = 3·37 0·1% = 4·59
			267 A	78·8±1·49		89·4±0·88	
			267 B	83·3±1·79		89·6±0·72	
			267 C	65·9±1·79		85·6±1·01	
5	23–25	46–58	263	83·5±2·49	5% = 3·79 1% = 5·20 0·1% = 7·08	92·7±0·73	5% = 1·61 1% = 2·20 0·1% = 3·00
			263 A	84·5±1·51		89·4±0·74	
			267 D	71·2±1·99		87·8±0·87	
			267 E	75·7±1·31		88·2±0·60	
6	25	54–61	263	84·5±3·05	5% = 5·63 1% = 7·72 0·1% = 10·51	91·2±0·70	5% = 1·85 1% = 2·53 0·1% = 3·45
			282	76·4±2·69		91·4±0·76	
			267 F	67·5±1·22		86·0±0·77	
			283	80·1±2·43		89·0±0·72	
7	23–24	49–61	263	87·6±1·73	5% = 2·78 1% = 3·81 0·1% = 5·19	92·7±0·66	5% = 1·99 1% = 2·72 0·1% = 3·71
			284	83·7±1·70		91·6±0·72	
			267 F	71·4±0·93		85·1±1·13	
			286	86·7±1·29		87·0±0·53	

* In all experiments except no. 2 an analysis of variance was carried out, for details see Table 5, the least mean differences necessary for significance at 5, 1 and 0·1% are quoted here. In Exp. 2 the paired *t*-test of 'Student' (*Biometrika*, 1908, **6**, 1; *Metron*, 1925, **5**, 105) was used. Comparisons were made between values obtained for each milk with the same rat. S.E.M. = standard error of the mean of the difference. *P* = probability that a mean difference at least as great as the observed mean difference would have arisen by random sampling from a homogeneous population.

The b.v. of 76·6 now obtained for the proteins of the deteriorated milk is higher than the previous value of 71·1 after 4½ years' storage (6, 7). This can probably be attributed to variations in response between different batches of rats, and emphasizes the advisability of including a standard of comparison in such tests.

In previous tests there was no indication of a decrease in the t.d. of the milk whose b.v. decreased on storage (6, 7). In the present experiment the t.d. proved inferior to that of the L control powder. The latter, moreover, was further increased by the addition of cystine, whereas no such benefit was conferred on the deteriorated sample. Changes in

t.d. of powders on the addition of cystine or lysine were also observed in Exps. 2, 6 and 7; these findings are more fully considered in the discussion (p. 352).

Effect of the addition of cystine to freeze-dried milk (Exp. 2)

Fairbanks & Mitchell (8) found that roller-drying at the lowest possible temperature, or spray-drying without preheating, did not impair the b.v. of liquid skim milk and that the dried product was improved by the addition of cystine. They concluded from growth tests that cystine was affected by the heating but did not carry out tests with cystine added to liquid skim milk.

Table 4. *Mean results for groups of 6 rats for the protein (N × 6·38) efficiencies of the milks studied in the various experiments*

Exp. no.	Age of rats at start (days)	Weight of rats at start (g.)	Diet no.	Diet intake (g.)	Gain in weight (g.)	Protein efficiency: Numerical result and its S.E. of the mean	Protein efficiency: Statistical significance*
3	33–38	94–124	259	369·35	83·0	2·78±0·075	S.E.M. ±0·12
			263	376·77	90·3	2·93±0·105	$P = 1:4$
4	22–25	46–73	267	289·21	67·5	2·83±0·062	5% = 0·30
			267A	264·24	50·0	2·28±0·053	1% = 0·41
			267B	271·52	53·8	2·38±0·183	0·1% = 0·57
			267C	225·10	26·0	1·41±0·161	
5	24–26	48–68	263	285·03	64·8	2·79±0·096	5% = 0·24
			263A	261·56	54·7	2·56±0·097	1% = 0·34
			267D	258·23	43·5	2·10±0·036	0·1% = 0·47
			267E	272·15	49·2	2·24±0·086	
6	25–26	63–69	263	273·82	61·2	2·81±0·037	5% = 0·19
			282	262·98	55·8	2·59±0·098	1% = 0·26
			267F	251·89	36·3	1·82±0·081	0·1% = 0·36
			283	279·01	57·3	2·55±0·069	
7	24–26	51–73	263	284·55	62·5	2·71±0·039	
			284	286·36	62·8	2·70±0·043	5% = 0·21
			282	293·77	62·2	2·60±0·080	1% = 0·29
			285	282·62	53·8	2·35±0·068	0·1% = 0·40
			267F	264·39	39·2	1·83±0·093	
			286	273·86	54·5	2·42±0·079	
8	24–26	48–72	263	281·28	62·7	2·76±0·076	
			290	299·52	68·0	2·80±0·076	5% = 0·37
			267G	224·66	26·5	1·47±0·046	1% = 0·51
			286A	247·92	38·8	1·92±0·158	0·1% = 0·70
			291	258·73	46·5	2·16±0·718	
			292	273·18	50·3	2·27±0·088	

* See footnote to Table 3. The paired *t*-test was used only in Exp. 3.

The experiment just described showed that cystine, in fact, limits the b.v. of freshly prepared dried milk. Mitchell & Block (9) concluded, from microbiological assays, that cystine and methionine were limiting amino-acids in the proteins of fresh milk.

To confirm that unheated milk was deficient in cystine, tests were carried out with freeze-dried milk, as this is easier to include in a diet than milk in the liquid state.

A sample of bulk milk from the herd of the National Institute for Research in Dairying was freeze-dried, after separation, by Dr R. A. Kekwick of the Lister Institute, London. The b.v. and t.d. of this milk, alone or with cystine added at the same rate as in Exp. 1, were determined by the method of Mitchell (1, 2). Owing to difficulties in drying a sufficient quantity of milk a parallel test by the rat-growth technique (3) was not included.

Table 5. *Analysis of variance of the data obtained in all experiments*

Component	Degrees of freedom	Sum of squares	Mean square	Variance ratio
		Exp. 1. Biological value		
(i) Total	47	4571·87	—	—
(ii) Litters	2	194·64	97·32	ii/viii 8·57*, ii/vi 2·19 N.S.
(iii) Diets	3	3036·13	1012·04	iii/viii 89·06†, iii/v 38·76†
(iv) Periods	3	21·31	7·10	iv/viii 1·60 N.S., iv/vi 6·25‡
(v) Litters × diets	6	156·70	26·12	v/viii 2·30 N.S.
(vi) Litters × periods	6	266·36	44·39	vi/viii 3·91*
(vii) Rats within litters	9	692·21	76·91	vii/viii 6·77†
(viii) Error	18	204·52	11·36	—
		True digestibility		
(i) Total	47	1022·07	—	—
(ii) Litters	2	38·96	19·48	ii/viii 2·11 N.S., ii/vi 4·60 N.S.
(iii) Diets	3	636·22	212·07	iii/viii 22·93†, iii/v 83·51†
(iv) Periods	3	52·94	17·65	iv/viii 1·91 N.S., iv/vi 4·16 N.S.
(v) Litters × diets	6	15·23	2·54	v/viii 3·64‡
(vi) Litters × periods	6	25·44	4·24	vi/viii 2·18 N.S.
(vii) Rats within litters	9	86·73	9·64	vii/viii 1·04 N.S.
(viii) Error	18	166·55	9·25	—
		Exp. 4. Biological value		
(i) Total	47	4407·38	—	—
(ii) Litters	2	63·85	31·93	ii/viii 1·54 N.S., ii/vi 3·15 N.S.
(iii) Diets	3	3026·90	1008·97	iii/viii 486·6†, iii/v 128·0†
(iv) Periods	3	493·47	164·49	iv/viii 79·36†, iv/vi 16·24*
(v) Litters × diets	6	47·25	7·88	v/viii 2·63 N.S.
(vi) Litters × periods	6	60·76	10·13	vi/viii 2·05 N.S.
(vii) Rats within litters	9	341·97	38·00	vii/viii 1·83 N.S.
(viii) Error	18	373·18	20·73	—
		True digestibility		
(i) Total	47	534·02	—	—
(ii) Litters	2	7·15	3·58	ii/viii 2·30 N.S., ii/vi 1·38 N.S.
(iii) Diets	3	185·21	61·74	iii/viii 7·50*, iii/v 19·60*
(iv) Periods	3	65·44	21·81	iv/viii 2·65 N.S., iv/vi 8·42‡
(v) Litters × diets	6	18·87	3·15	v/viii 2·61 N.S.
(vi) Litters × periods	6	15·54	2·59	vi/viii 2·62 N.S.
(vii) Rats within litters	9	93·63	10·40	vii/viii 1·26 N.S.
(viii) Error	18	148·18	8·23	—
		Protein efficiency		
(i) Total	23	7·7619	—	—
(ii) Litters	5	0·5301	0·1060	ii/iv 1·77 N.S.
(iii) Diets	3	6·3335	2·1112	iii/iv 35·24†
(iv) Error	15	0·8983	0·0599	—
		Exp. 5. Biological value		
(i) Total	47	3393·98	—	—
(ii) Litters	2	22·62	11·31	ii/viii 1·73 N.S., ii/vi 1·34 N.S.
(iii) Diets	3	526·81	175·60	iii/viii 8·99†, iii/v 1·01 N.S.
(iv) Periods	3	742·56	247·52	iv/viii 12·67†, iv/vi 29·25†
(v) Litters × diets	6	1045·81	174·30	v/viii 8·92†
(vi) Litters × periods	6	50·78	8·46	vi/viii 2·31 N.S.
(vii) Rats within litters	9	653·73	72·64	vii/viii 3·72*
(viii) Error	18	351·67	19·54	—
		True digestibility		
(i) Total	47	469·54	—	—
(ii) Litters	2	69·69	34·85	ii/viii 9·93*, ii/vi 6·70‡
(iii) Diets	3	180·15	60·05	iii/viii 17·11†, iii/v 9·49‡
(iv) Periods	3	31·87	10·62	iv/viii 3·03 N.S., iv/vi 2·04 N.S.
(v) Litters × diets	6	38·00	6·33	v/viii 1·80 N.S.
(vi) Litters × periods	6	31·21	5·20	vi/viii 1·48 N.S.
(vii) Rats within litters	9	55·43	6·16	vii/viii 1·75 N.S.
(viii) Error	18	63·19	3·51	—

Table 5 (*continued*)

Component	Degrees of freedom	Sum of squares	Mean square	Variance ratio
		Protein efficiency		
(i) Total	23	2·5743	—	—
(ii) Litters	5	0·2331	0·0466	ii/iv 1·18 N.S.
(iii) Diets	3	1·7504	0·5835	iii/iv 14·81†
(iv) Error	15	0·5908	0·0394	—
		Exp. 6. Biological value		
(i) Total	47	5026·48	—	—
(ii) Litters	2	104·04	52·02	ii/viii 1·21 N.S., ii/vi 2·72 N.S.
(iii) Diets	3	1871·57	623·86	iii/viii 14·48†, iii/v 37·58†
(iv) Periods	3	1469·80	489·93	iv/viii 11·37†, iv/vi 25·60†
(v) Litters × diets	6	99·62	16·60	v/viii 2·60 N.S.
(vi) Litters × periods	6	114·81	19·14	vi/viii 2·25 N.S.
(vii) Rats within litters	9	591·09	65·67	vii/viii 1·52 N.S.
(viii) Error	18	775·55	43·08	—
		True digestibility		
(i) Total	47	518·29	—	—
(ii) Litters	2	84·63	42·32	ii/viii 9·20*, ii/vi 7·23‡
(iii) Diets	3	229·70	76·57	iii/viii 16·64†, iii/v 31·38†
(iv) Periods	3	0·99	0·33	iv/viii 13·94†, iv/vi 17·73*
(v) Litters × diets	6	14·64	2·44	v/viii 1·89 N.S.
(vi) Litters × periods	6	35·07	5·85	vi/viii 1·27 N.S.
(vii) Rats within litters	9	70·47	7·83	vii/viii 1·70 N.S.
(viii) Error	18	82·79	4·60	—
		Protein efficiency		
(i) Total	23	4·0143	—	—
(ii) Litters	5	0·3033	0·0607	ii/iv 2·54 N.S.
(iii) Diets	3	3·3522	1·1174	iii/iv 46·73†
(iv) Error	15	0·3588	0·0239	—
		Exp. 7. Biological value		
(i) Total	47	3125·91	—	—
(ii) Litters	2	88·99	44·50	ii/viii 4·24‡, ii/vi 3·82 N.S.
(iii) Diets	3	2016·17	672·06	iii/viii 64·01†, iii/v 15·83*
(iv) Periods	3	308·90	102·97	iv/viii 9·81†, iv/vi 8·83‡
(v) Litters × diets	6	254·78	42·46	v/viii 4·04*
(vi) Litters × periods	6	69·96	11·66	vi/viii 1·11 N.S.
(vii) Rats within litters	9	198·16	22·02	vii/viii 2·09 N.S.
(viii) Error	18	188·95	10·50	—
		True digestibility		
(i) Total	47	804·19	—	—
(ii) Litters	2	48·33	24·17	ii/viii 4·51‡, ii/vi 6·89‡
(iii) Diets	3	473·70	157·90	iii/viii 29·45†, iii/v 30·37†
(iv) Periods	3	5·74	1·91	iv/viii 2·81 N.S., iv/vi 1·84 N.S.
(v) Litters × diets	6	31·22	5·20	v/viii 1·03 N.S.
(vi) Litters × periods	6	21·04	3·51	vi/viii 1·53 N.S.
(vii) Rats within litters	9	127·66	14·18	vii/viii 2·65 N.S.
(viii) Error	18	96·50	5·36	—
		Protein efficiency		
(i) Total	35	4·1591	—	—
(ii) Litters	5	0·1331	0·0266	ii/iv 0·89 N.S.
(iii) Diets	5	3·2858	0·6572	iii/iv 22·20†
(iv) Error	25	0·7402	0·0296	—
		Exp. 8. Protein efficiency		
(i) Total	35	10·6341	—	—
(ii) Litters	5	0·7135	0·1427	ii/iv 1·60 N.S.
(iii) Diets	5	7·6936	1·5387	iii/iv 17·28†
(iv) Error	25	2·2270	0·0891	—

*=significant at 1%. †=significant at 0·1%.
‡=significant at 5%. N.S.=not significant.

The results of this experiment are shown in Table 3. With the addition of cystine, the b.v. increased from 90·1 to 97·3, the corresponding increase in t.d. was from 92·5 to 94·5; both increases were statistically significant.

These results confirm the view that cystine is a limiting factor in milk proteins, but suggest that this amino-acid is not affected by moderate heat treatment.

Comparison of the freshly prepared low- and medium-moisture control powders (*Exp.* 3)

A comparison was carried out, by the rat-growth method (3), of the freshly prepared samples of L and M powders in order to check that no marked differences existed between the nutritive values of their proteins (cf. p. 341). The results of this experiment are shown in Table 4. The values of 2·78 and 2·93 obtained for the p.e. of the L and M milks respectively are very similar and statistically indistinguishable. It may, therefore, be concluded that, as regards the quality of their proteins, no differences existed initially between the L, M and H powders.

Effect of storage at 37° C. *on the high-moisture powder* (*Exp.* 4)

The following H milks stored at 37° C. were selected, and the nutritive value of their proteins determined by both the balance-sheet (1, 2) and the rat-growth (3) methods: (1) Stored 8 days, air-packed; the flavour of this powder had seriously deteriorated but it was normal in other respects except for 'caking' due to crystallization of the lactose. (2) and (3) Stored 28 days, air-packed and gas-packed respectively; both powders showed marked loss of solubility at 20° C. and a slight loss at 50° C.; all oxygen was absorbed in the air-packed sample, i.e. there was maximum difference between air- and gas-pack. (4) Stored 85 days in air with resulting maximum development of insolubility.

A control sample was not included in this test. It was only possible to test four powders simultaneously by Mitchell's method (1, 2) and, on balance, it was considered that the four milks selected would yield the maximum information about the effects of storage.

The results of this experiment will be found in Tables 3, 4 and 5. The air-packed sample stored for 8 days had a b.v. of 86·9. This figure is somewhat lower than that obtained for the L control powder in Exp. 1, but it is higher than the values for the M control powder in Exps. 5 and 6, and almost the same as in Exp. 7. It may therefore be concluded that no deterioration in the b.v. had occurred after this period of storage. Storage of air-packed samples for 28 and 85 days resulted in a progressive and statistically significant lowering of the b.v. of the milk proteins to 78·8 and 65·9 respectively. The value of 83·3 obtained for the 28-day gas-packed sample was statistically superior to that found for the corresponding air-packed sample; it was lower, but not significantly so, than the figure found for the 8-day air-packed powder. It thus becomes evident that the changes on storage in the milk proteins are retarded under anaerobic conditions. The t.d. of the sample stored for 85 days was, statistically, markedly inferior to that of the other three milks, among which the small differences were not statistically significant.

There was a definite parallelism between the results obtained for the p.e. (Table 4) and those for the b.v. (Table 3), but the differences were less marked by the former method. This was particularly noticeable with the 28-day air- and gas-packed samples where the difference was not statistically significant.

Effect of moisture content and of temperature of storage on deterioration in samples of powder after 6 months' storage (Exp. 5)

After 6 months' storage at 37° C. M powder was showing signs of mild deterioration, as judged by chemical tests, while H powder stored at 28·5° C. was showing more marked changes. Tests were therefore carried out on M control powder, on air-packed M powder stored at 37° C. for 6 months, and on H powder stored at 28·5° C. for 6 months, air- and gas-packed. This milk was tested in order to confirm the difference observed in Exp. 4 between air- and gas-packed samples. The tests were carried out by the nitrogen-balance (1, 2) and the growth (3) methods.

The results are given in Tables 3–5. There was no decrease in the b.v. of M powder stored for 6 months at 37° C., but the t.d. showed a statistically significant decrease from 92·4 to 89·4. The air- and gas-packed samples of H powder stored for 6 months at 28·5° C. showed a significant decrease in both the b.v. and the t.d. The drop in b.v. was again more marked for the air- than for the gas-packed sample.

The results of the growth test again roughly confirmed those obtained by the balance-sheet method but the differences lacked the same degree of significance.

Effect of the addition of 2·5% lysine to the deteriorated high-moisture powder and to control powder (Exp. 6)

In chemical tests Van Slyke estimations and formol titration indicated a change in the deteriorated milk powders whereby the free ϵ-amino-group of part of the lysine in the milk became blocked by a reaction, probably with carbohydrate (Part III). It seemed likely that lysine altered in this way would not be fully available to the rat, and that this change might be largely responsible for the lowered nutritive value of the milk. The two chemical methods did not indicate the same loss of amino-groups, the Van Slyke method showing a drop of some 60% during the storage of H powder at 37° C. for 60 days, the formol titration, of very much less (Part III). The results of the microbiological tests (Part V) were not yet available. In order to ensure full replacement of any lysine inactivated during storage, pure lysine was added to a control and to the deteriorated sample of milk at the rate of 2·5%, i.e. at the approximate original concentration ((10, 11, 12), Part V).

L-Lysine was added as the monohydrochloride dihydrate to M control powder and to a gas-packed sample of H powder, stored at 37° C. for 60 days. These mixtures were then analysed for N and made into diets to supply approximately 8% protein (cf. p. 341). Tests were carried out by both the balance-sheet (1, 2) and the rat-growth (3) techniques. The results are given in Tables 3–5.

It will be seen that both the b.v. and the p.e. of the deteriorated milk were markedly improved by the addition of 2·5% lysine. In neither case were the values obtained as high as those for the control milk; this difference was statistically significant for the p.e. but not for the b.v. The t.d. of the deteriorated powder was markedly lower than that of the control powder; the addition of the lysine, even if allowance is made for its complete digestibility, caused a statistically significant increase in the t.d., but the higher value obtained was still statistically inferior to that for the control powder.

The lowering of both the b.v. and the p.e. of the control powder by the addition of lysine is, at first sight, somewhat surprising. It is known that lysine is in no way a limiting

factor in milk(13) and this amino-acid, together with valine, is believed to be chiefly responsible for the supplementary relationship between the proteins of milk and wheat(14), the latter food being deficient in these amino-acids. Riesen, Schweigert & Elvehjem(15) noted an improvement in the efficiency of nitrogen utilization by rats when 0·22 L-cystine or 0·4% DL-methionine were added to casein, but little or no improvement when the two amino-acids were added together. They suggested that this finding might be due to an imbalance of amino-acids when the two acids were added simultaneously. Somewhat similar observations were made in human experiments by Murlin, Edwards, Hawley & Clark(16) who studied the effects of added amino-acids on the biological value of egg- and soya bean-proteins. It is possible that such an effect may account for our findings. On the other hand, as milk contains adequate amounts of lysine, it may be that the rat simply does not utilize the amino-acid added to the control powder, and that this nitrogen is excreted in the urine. The b.v. and the p.e. values for the control powder to which lysine had been added were therefore recalculated on the assumption that the lysine nitrogen was not utilized. For this purpose the lysine nitrogen was subtracted from the urinary nitrogen in the measurements by the Mitchell method(1, 2) or from the nitrogen intake in measurements by the growth technique(3). The values of 84·3 and 2·85, respectively, so obtained are very close to the corresponding figures of 84·5 and 2·81 obtained for the control milk alone.

The results of the microbiological assays (Part V) were available when this experiment was completed. They indicated that, in H powder stored in air-pack at 37° C. for 95 days, some 40% of the lysine originally present resisted enzymic hydrolysis. This suggested that in the experiment just described, an excess of lysine had been added even to the deteriorated powder and that this excess may have prevented the b.v. and the p.e. of the supplemented deteriorated powder from reaching those of the control powder. The alternative explanation that, besides lysine, other essential amino-acids had been affected was by no means ruled out but, before this possibility was investigated, further tests were done with smaller additions of lysine.

Effect of the addition of 1·25% *lysine to the deteriorated high-moisture powder, and of lysine at several levels to the control powder* (*Exp.* 7)

As mentioned above, microbiological tests indicated that rather less than 50% of the lysine originally present in milk had, after storage, become resistant to enzymic hydrolysis. It was decided, therefore, to add 1·25% lysine to a deteriorated powder. If lysine was the only amino-acid affected, its addition at this rate should cause full restoration to the initial b.v. At the same time it was considered that a study of the addition of graded amounts of lysine to the control powder would be profitable. It was not practicable to test more than six samples of powder and the following were chosen: (1) M control; (2) M control with 1·25% lysine; (3) M control with 2·5% lysine; (4) M control with 5% lysine; (5) H powder, gas-packed ,stored for 60 days at 37° C.; (6) H powder, gas-packed, stored for 60 days at 37° C., with 1·25% lysine. In making the diets allowance was made for the nitrogen supplied by lysine (cf. Exp. 6). All samples were tested by the growth method(3); in addition, samples (1), (2), (5) and (6) were tested by the balance-sheet method(1, 2). The results of this experiment are shown in Tables 3–5.

The addition of 1·25% lysine to the deteriorated powder raised its b.v. almost to the level of the control powder, the difference between these two being without statistical

significance. The addition of lysine to the control powder again caused a significant lowering of the b.v. If it is assumed that this added lysine was not required by the rats, a recalculation of the b.v. gives a value of 88·0, almost identical with the value of 87·6 obtained for the M control powder. This finding lends strong support to the belief, expressed above, that the lowered b.v. of high-quality powder observed after the addition of lysine is due to the superfluity of the latter. The matter is being put to a more direct test.

The t.d. of the deteriorated powder was found to have fallen, as in previous experiments; the addition of 1·25% lysine caused an increase which was not statistically significant.

The figures for the p.e. again followed the same trend as those obtained for the b.v. but, as in the earlier experiments, the differences were less marked. The addition of 1·25% lysine to the deteriorated powder caused a marked increase, but the value obtained was still inferior to that found for M control powder. In this test the addition of 1·25 and 2·5% lysine to M control powder had little or no effect on its p.e., but 5% lysine caused a significant lowering. If it is again assumed that this lysine was in excess of the animals' requirements, and allowance is made for this, the value of 2·35 becomes 2·73, almost identical with that of 2·71 obtained for the M control powder; a similar calculation gives a value of 2·81 for the powders with the addition of 1·25 and 2·5% lysine.

Effect of the addition of lysine, histidine and arginine to the deteriorated high-moisture powder (Exp. 8)

It was found in Exp. 7 that, although the addition of 1·25% lysine to the deteriorated powder largely restored the b.v. to its initial value, the improvement in the t.d. and p.e. was less satisfactory. The results of the microbiological assay (Part V) suggested that histidine, arginine, and possibly methionine might also have been affected, although much less than lysine. Supplies of the powders originally prepared for these tests were running low and it was not possible to carry out tests by both the balance-sheet (1, 2) and the growth (3) techniques; in addition only a sample of H powder which had been stored at 37° C. for 60 days in the presence of a relatively large supply of air (cf. Part III, p. 309) was available. It had been found in Exps. 4 and 5 that deterioration was more marked in air-pack than in gas-pack. A preliminary test was carried out using only the less laborious growth method (3) to study the effects of the addition of histidine and arginine as well as lysine to the deteriorated powder. L-Arginine and L-histidine were added to the powders as the monohydrochlorides at the rate of 0·5%, that is about 30% of the amount of these acids originally present, as the microbiological tests indicated a possible loss of this order (Part V). The following samples of powder were tested: (1) M control; (2) M control with 0·5% histidine and 0·5% arginine; (3) H powder air-packed, stored for 60 days at 37° C.; (4) H powder, air-packed, stored for 60 days at 37° C., with 1·25% lysine; (5) H powder, air-packed, stored for 60 days at 37° C., with 1·25% lysine and 0·5% histidine; (6) H powder, air-packed, stored for 60 days at 37° C., with 1·25% lysine, 0·5% histidine and 0·5% arginine. The results of this experiment are shown in Tables 4 and 5.

It will be seen that the addition of histidine and of arginine to the deteriorated powder supplemented with lysine caused an improvement in the p.e. which, though suggestive, just lacked conventional statistical significance. The value obtained with these three

amino-acids still fell short of that observed for the M control powder. Nevertheless, the result suggested that this further addition of histidine and arginine to the deteriorated powder supplemented with lysine had had a beneficial effect. It should be noted that the addition of histidine and arginine did not depress the p.e. of the control powder, although the total addition of these acids amounted to 1%. This is in line with the finding that the addition of 1·25% lysine did not depress the p.e. of the control milk although it depressed its b.v. (Exp. 7). It is hoped to repeat this experiment at a later date using Mitchell's method (1, 2). It must be borne in mind that the deteriorated sample used in this experiment was packed in air, and not in nitrogen as in Exp. 7, and that the amount of oxygen which had been absorbed by this powder was some three times greater than with the air-packed samples tested in Exps. 4 and 5.

DISCUSSION

The experiments described above confirm our earlier findings (6, 7) that the proteins of dried skim milk undergo marked deterioration in nutritive value on storage. They show that the moisture content of the powder has a determining influence in the development of this fault. The lowering of the nutritive value of milk proteins was chiefly due to changes in the availability of lysine. In fact, if the probable loss of this acid, as judged by amino-nitrogen measurements (Part III) and by microbiological assay after enzymic hydrolysis (Part V), was made good, the b.v. of a gas-stored deteriorated powder could be restored to its original value (Exp. 7). On the other hand, this addition of lysine only partly restored the p.e. of the powder. It stands to reason that the latter value will be influenced, to some extent at any rate, by the lowered digestibility of the proteins of deteriorated powder and that, in judging the efficiency of its proteins to promote growth, only the value of that part which is available should be considered. Table 6 gives such

Table 6. *Protein efficiencies of the deteriorated milks stored at* 37° C. *for* 60 *days, supplemented with amino-acids, recalculated from values given in Table* 4 *by making allowance for the lowered digestibility of the milk proteins as indicated by findings in tests by the balance-sheet method quoted in Table* 3.

Exp. no.	Type of packing	Supplement	Value found	'Corrected' value*	Value for M control milk
6	Gas	2·5% lysine	2·55	2·74	2·81
7		1·25% lysine	2·42	2·60	2·71
8	Air	1·25% lysine	1·92	2·07	2·76
8		1·25% lysine + 0·5% histidine	2·16	2·32	2·76
8		1·25% lysine + 0·5% histidine + 0·5% arginine	2·27	2·44	2·76

* In Exps. 6 and 7 the t.d. of the deteriorated milk was only 93% of that of the original milk as measured by the parallel Mitchell (1, 2) test, the same figure was assumed for Exp. 8 where no Mitchell tests were carried out.

'corrected' values, based on t.d. found by the Mitchell technique in Exps. 6 and 7. These calculations show that for Exps. 6 and 7, where a gas-packed sample of powder was used, the p.e. was largely restored to the value obtained for M control powder; with the air-packed sample used in Exp. 8 the gap between the 'corrected' and the 'control' figures still remained appreciable. This suggests that, for air-stored powder at least, a further amino-acid or acids, in addition to lysine, is involved, and is in line with the findings of Exps. 4 and 5 where the b.v. of milk deteriorated more markedly on storage in an air-pack than when gas-packing was used.

It will have been noticed that on several occasions the addition of an amino-acid to a milk powder increased not only its b.v. but also its t.d. It is not clear how this second effect is brought about and why the absorbability of a protein should be altered by the addition to it of an amino-acid. To a certain extent the change is only apparent as the added amino-acid is, no doubt, 100% digestible, and this influences the value for the t.d. of the mixture. By allowing for this influence, it can be calculated, for example, that in Exp. 6 the t.d. of the deteriorated powder to which 2·5% lysine had been added should be 88·1 and not 89·0. This corrected value is no longer significantly different from that (86·0) for the unsupplemented powder. In this case, therefore, the improvement was most probably fortuitous. Confirmation of this is found in Exp. 7 where the addition of 1·25% lysine to the deteriorated powder increased the t.d. from 85·1 to 87·0; this difference was, however, below the conventional level of significance and, when allowance is made for the complete absorption of the lysine nitrogen, the corrected figure of 86·3 is, of course, statistically indistinguishable from the original value. The increase in t.d. of freshly prepared powders after the addition of cystine in Exps. 1 and 2 cannot, however, be explained in the same way, as the amount of cystine nitrogen was relatively very small and allowance for it does not alter the statistical significance of the difference. It is of interest that the t.d. of the deteriorated sample was not affected by the addition of cystine, whereas lysine caused an apparent improvement only with the deteriorated powders.

The results of Exp. 8 suggest that, in addition to lysine, histidine, alone or in combination with arginine, might completely restore the nutritive value of the proteins of deteriorated gas-packed powder. As shown below, the necessity of adding arginine is doubtful on theoretical grounds, and in effect it caused only an insignificant improvement when added in combination with histidine and lysine. It has been shown by Scull & Rose (17) that the rat is able to synthesize arginine but the rate of synthesis is not sufficient to maintain normal growth and the presence of dietary arginine is essential (18). Table 7

Table 7. *Percentages of essential amino-acids in the experimental diets (containing 23% dried milk) calculated on the basis of the microbiological data (Part V) for undeteriorated control milk, compared with Rose's (22) figures for the quantities needed to satisfy minimum requirements for normal growth of rats*

Amino-acid	Calculated amount in diet (%)	Rose's minimum requirements (%)
Arginine	0·32	0·2
Histidine	0·32	0·4
Isoleucine	0·48	0·5
Leucine	1·53	0·9
Lysine	0·61	1·0
Methionine	0·22	0·6
Phenylalanine	0·37	0·7
Threonine	0·59	0·6
Tryptophan	0·10	0·2
Valine	0·56	0·7
Cystine	0·05	—

compares the approximate amounts of the essential amino-acids, likely to be available in the control milk diets fed to rats in the present experiments, with Rose's (18) figures for the minimum amounts needed for normal growth when non-essential acids are included in the diet. It will be seen from this table that only arginine and leucine were supplied in excess of the animals' minimum requirements for individual amino-acids. It should be noted, however, that at the 8% level of protein intake at which our tests were done, the

growth rate of rats is subnormal and that their requirements would be below the minimum values quoted in Table 7.

Even a 30% loss of arginine still leaves this acid at approximately the minimum figure postulated by Rose (18). It therefore seems unlikely that addition of arginine would appreciably enhance the nutritive value of the proteins of the deteriorated powder.

Briefly stated, the biological tests give the following picture of the changes in milk powder of high moisture content brought about by storage. A loss or inactivation of lysine accounts for the bulk of the depression of the b.v.; this may be accompanied or followed by a slight loss of histidine. Inactivation of lysine is probably dependent on the atmosphere in the container only in so far as the reaction proceeds more rapidly after the crystallization of lactose which, in these experiments, occurred slightly earlier in the air- than in the gas-packed powders (Part III, p. 324). The loss of histidine may require, or be increased by, the presence of oxygen, but further work is necessary to confirm the loss of this acid and to ascertain the influence of oxygen on the process. The microbiological tests (Part V) indicate the possibility of a slight loss of methionine during storage, but tests with this amino-acid have not yet been made with rats. The overriding influence of the moisture content of the powder on the inactivation of lysine has already been stressed (p. 338); it is not possible to say at present what is the influence of moisture content on the inactivation of acids other than lysine.

It is not yet known whether milks of low moisture content are also liable to these oxidative changes. None of the low-moisture milks were tested biologically because chemical and physical tests (Part III) indicated little or no change.

It has already been mentioned that good parallelism was observed between the results for the b.v. of the milk powder obtained by the balance-sheet method (1, 2) and the p.e. measured in *ad lib.* feeding tests by the growth technique of Osborne, Mendel & Ferry (3). The correlation between the results obtained by the two methods is shown graphically in Fig. 1. The agreement is excellent as indicated by the high correlation coefficient of 0·89, and there is no doubt that, in our experience at any rate, the simple growth technique has proved most valuable in assessing the quality of a protein. It is rather less sensitive to small differences than the balance-sheet method (1, 2), but is specially useful in pilot experiments.

Our findings are in agreement with results obtained by Carlson, Hafner & Hayward (14) who applied both these methods. When they used equalized feeding for the p.e. measurements, agreement with Mitchell's method (1, 2) was rather less satisfactory. Barnes, Maack, Knights & Burr (19) found that, for good quality proteins with a p.e. of about 2·0, *ad lib.* feeding tests gave, at a 10% level of protein intake, more uniform results than equalized feeding tests; lower results were obtained by the latter technique. They criticize the method strongly because it does not take into account the digestibility of the proteins tested. Mitchell (20) is of the opinion that the balance-sheet method (1, 2) is more accurate and detects smaller differences than the growth method (3), and we would be inclined to support this view. Mitchell (20) shows further that *ad lib.* feeding in the growth method may be expected, on account of its large experimental error, to exaggerate quality differences among proteins when these are large and to obscure them when they are small. He found that the food intake was usually greater for the better quality proteins; this was to some extent confirmed by the present experiments. Reference to Table 4 shows that the diet intake was, as a rule, greater with milks of higher p.e., but on three

occasions (diets 263A and 267D, Exp. 5; diets 263 and 283, Exp. 6; diets 263 and 285, Exp. 7) the difference was either negligible or in the other direction, though in all three cases the differences in p.e. were statistically significant.

Graves (21, 22) has strongly criticized the conclusions of Chick (23) based on tests by the growth method (3), contending that her results could have been predicted from the diet intakes of the rats. Chick's results were subsequently confirmed in this laboratory (24) by the balance-sheet method (1, 2). Graves (22) also criticized, on the same score, certain conclusions reached by Henry & Kon (25). This criticism is vitiated by the unwarranted

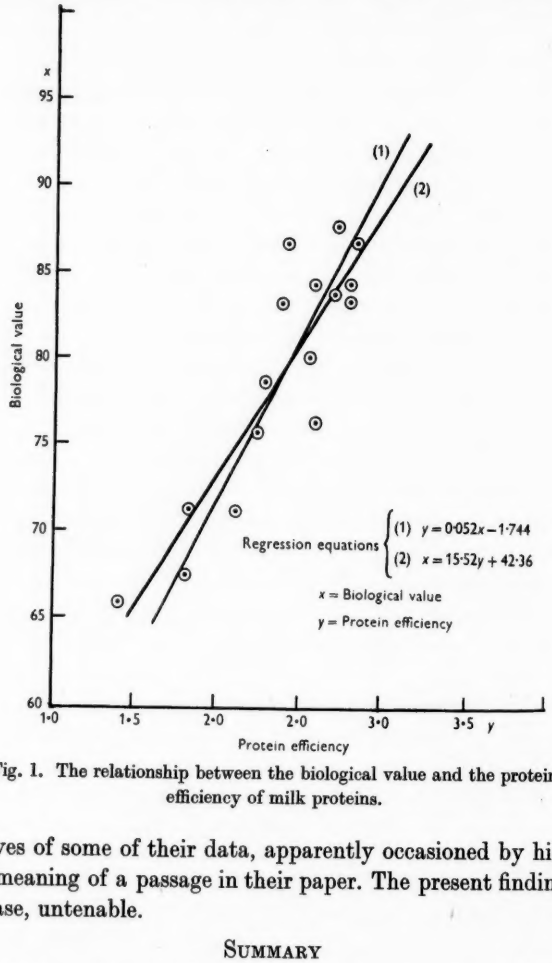

Fig. 1. The relationship between the biological value and the protein efficiency of milk proteins.

rejection by Graves of some of their data, apparently occasioned by his incorrect interpretation of the meaning of a passage in their paper. The present findings make his contention, in any case, untenable.

Summary

1. To investigate the causes and nature of the deterioration in nutritive value on storage of the proteins of dried skim milk (6, 7), biological tests with rats were carried out with a number of powders of different moisture content stored under different conditions. The experiments were done by the balance-sheet method of Mitchell (1, 2) and by the growth method of Osborne, Mendel & Ferry (3). Suitable samples of powder were chosen for this purpose on the basis of the chemical tests described in Part III of this paper.

2. The biological value of the proteins of dried skim milk of high moisture content (7·6%) decreased progressively during storage in air at 37° C.. Storage for 8 days produced no perceptible change (b.v. 86·9) but the value was 78·8 after 28 days and 65·9 after 85 days.

3. Under these conditions the true digestibility of the milk proteins did not alter after storage for 1 month, but a statistically significant decrease of 5–6% occurred by the end of 2 months; little change was observed after longer periods of storage.

4. At the lower storage temperature of 28·5° C. the change in high-moisture samples was about six times slower than at 37° C., the biological value and true digestibility of the proteins of a sample stored for 6 months being comparable with those of the sample stored for 1 month at 37° C.

5. Powder with a lower moisture content (5%) proved much more stable; the biological value of its proteins was unchanged after storage for 6 months at 37° C. though the true digestibility of the proteins decreased significantly by 4%.

6. Dried skim milks with the still lower content of 3% moisture were not examined, after storage, in these biological tests, as chemical tests showed that they suffered little deterioration under any conditions of storage.

7. The decrease in the biological value of the proteins was slightly more marked in air-packed than in gas-packed samples of milk. The true digestibility of the proteins was not affected by the type of packing.

8. Although differences were, in general, less marked, the values for the protein efficiency of the milks followed the same trend as those for the biological value of the proteins. The agreement between the two methods was good as shown by the high correlation coefficient of 0·89 for all results.

9. The addition of 1·25% lysine to a sample of high-moisture powder stored for 60 days at 37° C. in nitrogen, increased the biological value of the proteins approximately to the figure obtained for a control sample. An increase in the true digestibility of the proteins and in the protein efficiency of the milk was also observed but the values obtained fell short of those for the control milk.

10. The addition of histidine as well as lysine caused a further slight improvement in the protein efficiency of a sample of deteriorated milk which had been stored for 60 days at 37° C. in air, but arginine was without effect.

11. The addition of lysine to the control powder led to a significant lowering in both the biological value of the proteins and the protein efficiency. This was most probably not because the added lysine was harmful but because, given in excess, it was not utilized.

12. The addition of cystine to fresh samples of spray-dried and of freeze-dried skim milk significantly improved both the biological value and the true digestibility of the proteins of these milks.

13. The results thus indicate that a high moisture content in dried skim milk is the most important factor in the deterioration of the milk proteins. Most of the decrease in the biological value of the proteins of such milks is accounted for by the loss or inactivation of lysine; it is probable that some loss of histidine also occurs.

We are indebted to Prof. A. C. Chibnall, F.R.S., to Dr W. E. Gaunt and to Dr S. J. Rowland for helpful advice. Our thanks are due to Dr R. A. Kekwick for the preparation of the freeze-dried milk; to Miss M. V. Chapman for help with the care of the experimental animals and to Miss J. Wagnell for help with some of the nitrogen analyses.

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PART V. MICROBIOLOGICAL ASSAY OF 'ESSENTIAL' AMINO-ACIDS

By KATHLEEN M. HENRY, S. K. KON, C. H. LEA and J. C. D. WHITE

(With 1 Figure)

For the estimation of the ten amino-acids regarded as essential for the rat and of cystine, a control milk powder and six samples of deteriorated powder were selected. The deteriorated powders were all of high moisture content (H powder, cf. Part II), and had been stored at 37° C. for 10 days (air), 30 days (air), 30 days (gas) and 95 days (air), or at 28·5° C. for 176 days (air) and 176 days (gas) respectively. Samples were chosen to correspond as closely as possible to those which had been tested biologically as described in Part IV.

Method

The analyses were carried out for the authors by Dr E. C. Barton-Wright who used the methods of microbiological assay described by him (1). Since preliminary tests for several individual amino-acids carried out after hydrolysis of the powder showed little or no loss even in badly deteriorated samples, it was decided to apply enzymic hydrolysis with pancreatin, and to use acid hydrolysis of the control and of one badly deteriorated sample for purpose of comparison only.

There are several disadvantages in the use of the enzymic method, the most serious being cessation of hydrolysis before the protein is completely broken down to amino-acids. In the present work recovery of the eleven acids after treatment of the powders with pancreatin was only of the order of 55–60% of the recovery obtained after hydrolysis with acid (Table 1). In order to minimize errors introduced by inequalities in the degree of hydrolysis undergone by the various samples (the control, for example, showed an appreciably higher total recovery of acids than any of the stored samples) the results for individual acids have been recalculated in Table 2 as a percentage of the total amount of the eleven acids recovered. This device should serve to expose selective loss of a particular acid or acids. In the event of such loss the percentage of the remaining acids in the mixture would, of course, rise slightly in compensation. Since most of the chemical criteria measured (Part III) indicate that storage for 176 days at 28·5° C. is roughly equivalent to 30 (or perhaps slightly more) days at 37° C.—a result with which the nutritional data (Part IV) are also in agreement—the 28·5° C. samples have been included with those stored at 37° C. at an appropriate place in the tables. For the preparation of Fig. 1, the 30 days 37° C. and 176 days 28·5° C. samples stored in air and in nitrogen have been treated as replicates, and the four values averaged.

RESULTS

The error inherent in the estimation of 'essential' amino-acids in proteins by the microbiological method has been given as ±15% (2), although individual workers frequently claim a degree of reproducibility considerably higher than this. In the present work the carrying out of the assays directly on milk powder, and the special difficulties introduced by the necessity for employing enzymic hydrolysis militated against obtaining maximum accuracy. Within the limitations of the methods used, it can be seen that deterioration of the powder during storage caused no loss of threonine, leucine, valine, isoleucine, phenylalanine, tryptophan or cystine, whether hydrolysis was by acid or by enzyme. Histidine showed a slight loss both by acid and by enzyme, and arginine and methionine a slight loss by enzyme but not by acid (Table 2, Fig. 1). These changes, however, were small in relation to the possible errors in the determinations, and must be considered as of doubtful significance. Only with lysine was there reasonably strong evidence of apparent loss during storage, this being considerably greater when measured after hydrolysis with enzyme than when acid hydrolysis was used. This observation is in agreement with results recently reported by Eldred & Rodney (3) for heat-damaged casein, in which the amount of free lysine released by enzyme was considerably reduced, although the amount liberated by acid hydrolysis was only very slightly reduced. In both forms of deterioration there is the

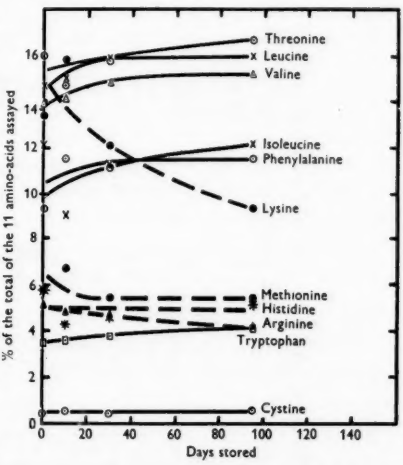

Fig. 1. Changes in content of 'essential' amino-acids during the storage of milk powder at 37° C. (enzymic hydrolysis).

Table 1. *Results of microbiological measurements of amino-acids in fresh and stored milk powders after hydrolysis with acid and with enzyme*

Days stored	Temp. (° C.)	Atmosphere	Method of hydrolysis	Amount of each acid found, as percentage of the dry powder									
				Lysine	Histidine	Arginine	Tryptophan	Phenylalanine	Leucine	Isoleucine	Valine	Threonine	Methionine	Cystine	Total
0	—	—	Acid*	2·65	1·40	1·37	0·45	1·60	3·02	2·10	2·43	2·58	0·94	0·22	18·76
95	37	Air	Acid*	2·04	1·10	1·34	0·40	1·45	2·95	1·90	2·22	2·54	0·86	0·20	17·00
0	—	—	Enzyme	1·70	0·74	0·65	0·44	1·18	1·86	1·53	1·76	2·03	0·73	0·05	12·67
10	37	Air	Enzyme	1·75	0·48	0·53	0·40	1·28	1·66	1·00	1·56	1·63	0·74	0·05	11·08
30	37	Air	Enzyme	1·50	0·48	0·53	0·43	1·30	1·66	1·03	1·57	1·81	0·70	0·05	11·06
176	28·5	N_2	Enzyme	1·30	0·58	0·52	0·46	1·28	1·94	1·40	1·76	1·66	0·51	0·05	11·46
176	28·5	Air	Enzyme	1·33	0·53	0·52	0·45	1·30	1·86	1·42	1·76	1·78	0·70	0·05	11·70
95	37	N_2	Enzyme	1·29	0·48	0·53	0·36	1·10	1·66	1·20	1·57	1·83	0·51	0·05	10·58
		Air	Enzyme	0·96	0·53	0·42	0·42	1·19	1·66	1·27	1·57	1·73	0·56	0·05	10·36

* Tryptophan was measured after hydrolysis with barium hydroxide.

Table 2. *Content of individual amino-acids measured microbiologically in milk powders, expressed as a percentage of the total of the eleven acids tested*

Days stored	Temp. (° C.)	Atmosphere	Method of hydrolysis	Lysine	Histidine	Arginine	Tryptophan	Phenylalanine	Leucine	Isoleucine	Valine	Threonine	Methionine	Cystine
0	—	—	Acid*	14·1	7·5	7·3	2·4	8·5	16·1	11·2	13·0	13·7	5·0	1·2
95	37	Air	Acid*	12·0	6·5	7·9	2·4	8·5	17·3	11·2	13·1	14·9	5·1	1·2
0	—	—	Enzyme	13·4	5·8	5·1	3·5	9·3	14·7	12·1	13·9	16·0	5·8	0·4
10	37	Air	Enzyme	15·8	4·3	4·8	3·6	11·5	15·0	9·0	14·1	14·7	6·7	0·5
30	37	Air	Enzyme	13·6	4·3	4·8	3·9	11·7	15·0	9·3	14·2	16·4	6·3	0·5
30	37	N_2	Enzyme	11·3	5·1	4·5	4·0	11·2	16·9	12·2	15·4	14·5	4·5	0·4
176	28·5	Air	Enzyme	11·4	4·5	4·4	3·9	11·1	15·9	12·1	15·0	15·2	6·0	0·4
176	28·5	N_2	Enzyme	12·2	4·5	5·0	3·4	10·4	15·7	11·3	14·8	17·3	4·8	0·5
Mean of 30 and 176 day values			Enzyme	12·1	4·6	4·7	3·8	11·1	15·9	11·2	14·8	15·8	5·4	0·4
95	37	Air	Enzyme	9·3	5·1	4·1	4·1	11·5	16·0	12·2	15·2	16·7	5·4	0·5

* Tryptophan was measured after hydrolysis with barium hydroxide.

suggestion of combination of the ε-amino-groups of the lysine residues in linkages more resistant to proteolytic enzymes than to acid.

Hodson & Krueger (4) have carried out microbiological tests of amino-acids after acid hydrolysis in freshly prepared dried skim milk and in samples which had been stored for 51 months at room temperature in air-tight cans or in cans with loose lids. They noted small losses of lysine, histidine, methionine and arginine in the samples stored in the loose-lidded cans.

SUMMARY

Microbiological tests of fresh and stored separated milk powders showed a definite apparent loss of lysine in deteriorated powder, the loss being greater when measured after enzymic, than after acid, hydrolysis. The maximum deficiency of lysine recorded was about 40% of the original content of this acid.

A slight loss of histidine was also probable, and of arginine and methionine possible, but the reproducibility of the methods was not sufficient to establish these with certainty.

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PART VI. GENERAL DISCUSSION

BY KATHLEEN M. HENRY, S. K. KON, C. H. LEA AND J. C. D. WHITE

(With 2 Figures)

Questions of physical and chemical changes, and of palatability, in the milk powders during storage, and those dealing with the work on the nutritive value of the proteins, and with the microbiological assay of amino-acids have already been discussed and summarized in the appropriate sections of Parts III, IV and V. There still remain to be considered, in relation to one another, the conclusions reached from the very different approaches to the problem which have been described in the foregoing parts.

The chemical data show that a reaction resulting in the destruction or 'blocking' of free amino-groups of the milk protein—which must consist largely of the ε-amino-groups of the lysine residues—occurs in powder of high moisture content during storage, particularly at high temperatures. Evidence is produced in support of the view that these changes originate from a reaction between the protein amino-groups and reducing sugar, very largely if not entirely, lactose. The data suggest, furthermore, that most of the objectionable physical and chemical changes in such powders, e.g. in palatability, colour and solubility, are connected with this reaction. Exclusion of atmospheric oxygen by packing in inert gas has little or no direct effect on the primary protein-sugar reaction, a small protective effect observed with nitrogen-packed powder being attributed to a slight retardation of crystallization of the lactose under these conditions. Subsequent degradation of the protein-sugar complex is influenced by the storage atmosphere, as is shown by the considerable absorption of oxygen by the air-packed material and by the more rapid production of carbon dioxide and development of 'off'-flavour under these

conditions. The protein-sugar reaction is profoundly influenced by the moisture content of the powder, and has a high temperature coefficient. At low moisture contents the reaction is almost completely prevented.

In addition to the primary protein-sugar reaction and to secondary changes resulting from it, evidence has been obtained, by gas exchange and palatability tests, of oxidative changes much less dependent on a high moisture content in the powder and possessing a much lower temperature coefficient. While the nature of these reactions has not been elucidated, it is probable that they include autoxidation of the trace of residual fat present in the powders.

Comparison of the chemical and biological data

The relation between the observed reduction in free amino-nitrogen and the loss of nutritive value of the proteins in the stored powders, as determined by the two biological methods used, is shown in Fig. 1. In the upper portion of the figure the biological values

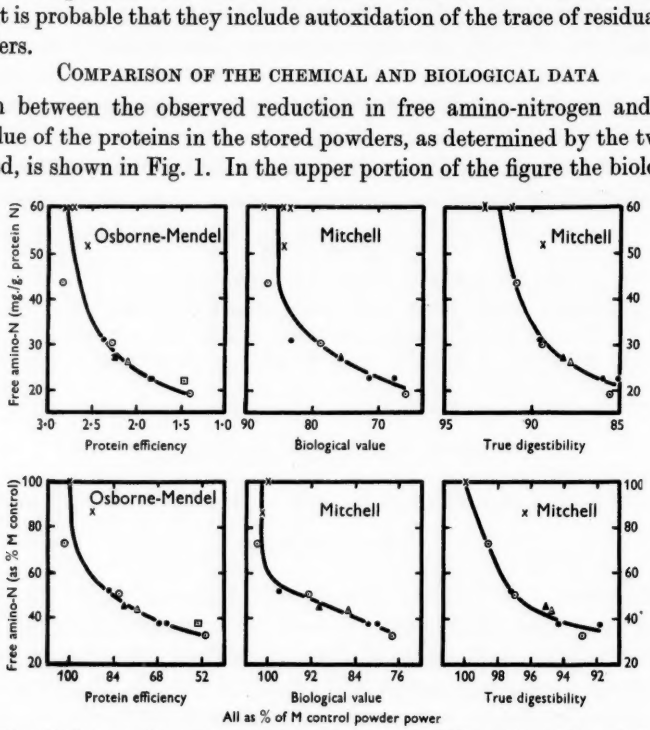

Fig. 1. The relationship between free amino-nitrogen content and nutritive value of the proteins. H powder, 37° C. air = ⊙; H powder, 37° C. 'extra air' = ⊡; H powder, 28·5° C. air = △; H powder, 37° C. nitrogen = ●; H powder, 28·5° C. nitrogen = ▲; M powder, control or 37° C. air = ×.

have been plotted as determined, the various data for M control powder obtained at different times with the different groups of rats being included separately. In the lower part of the figure all the values obtained in one experiment with one group of rats have been expressed as percentages of the value for the fresh, control powder in that experiment. For the one series which did not include a control, an average value based on all the available determinations was used.

The experimental points, which are largely derived from H powder stored in nitrogen or in a restricted supply of air at 37 or 28·5° C. for periods up to 6 months, lie on a reasonably smooth curve. This suggests that the blocking of free amino-groups of the protein leading to the inactivation of lysine was probably a major factor in producing the loss of nutritive value observed for these powders. The Osborne-Mendel and Mitchell data agree in showing little or no loss in nutritive value during the early stages of the protein-sugar

reaction, but a comparatively rapid loss later. This may be due to either or both of the following reasons. (1) Milk protein is rich in lysine, and it might be expected that this amino-acid would not become a limiting factor in determining the biological value of the protein until an appreciable proportion of it had been inactivated. (2) In the early stages of the protein-sugar reaction, when neither protein nor carbohydrate has undergone serious secondary change, it may be that the link between the ϵ-amino-groups of the lysine residues and sugar can be more easily split by the digestive enzymes with regeneration and utilization of part or all of the combined lysine. At a later stage of the process the regeneration of lysine may be more difficult or impossible.

With a deteriorated milk powder, determination of the free amino-nitrogen content of the intact protein yields values for lysine lower than those given by microbiological assay of lysine after enzymic hydrolysis of the protein (Part V) and these in turn are lower than values obtained in the same way after acid hydrolysis. The original lysine content of the fresh powder is never reached, however, and a rough estimate of the figures for a 37° C., 60-day, gas-stored H powder might be given as of the order of 40, 70 and 90% recovery respectively. This change in the recovery of lysine according to method of treatment suggests that the rat may be able to utilize a proportion of the bound lysine. In this

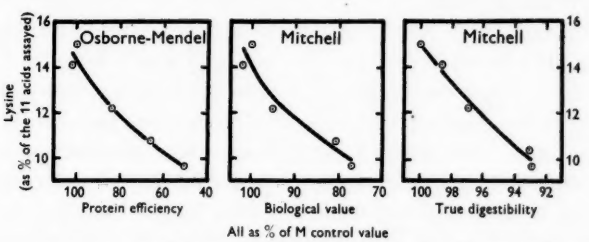

Fig. 2. The relationship between lysine content, as measured microbiologically after enzymic hydrolysis, and nutritive value of the proteins.

connexion it should be borne in mind that enzymic digestion prior to microbiological assay was continued for 5 days, and that Melnick, Oser & Weiss (1) have pointed out that for optimum utilization of food protein not only must all essential amino-acids be present, but they must also be liberated during digestion *in vivo* at rates permitting mutual supplementation. Omission of a single essential acid results in a poor utilization of the remainder which cannot be corrected for by injection of the missing acid some hours later (2). In the same way Henry & Kon (3) have demonstrated that supplementary relationships between two proteins only become evident when these are fed simultaneously and not when they are only available at different times.

Comparison of the results obtained in the rat-feeding experiments with those given by microbiological assay of lysine after enzymic hydrolysis of the deteriorated powders can only be made roughly, owing to the limitations of the microbiological data available. In Fig. 2, lysine values read off from Fig. 1, Part V, have been plotted against the nutritive value of the proteins as determined by the two biological methods. Only the biological results for H powder at 37° C. have been used, and data on air- and gas-stored powders have been averaged in order to bring them into line with the simplifications employed in deriving the lysine values. From Fig. 2 it can be seen that a much more direct relationship exists between loss of lysine, as determined by microbiological assay after enzymic

hydrolysis, and reduction in nutritive value, than between the latter and reduction in free amino-nitrogen (Fig. 1), although the curves are still somewhat bowed in the same direction as in Fig. 1. The implication is that in the deteriorated powder, part of the lysine which the free amino-values of the intact protein show to have combined, presumably with sugar, is still available to the rat, just as it is after enzymic hydrolysis to the bacteria used in the microbiological assay.

It appears from Figs. 1 and 2 that the addition of lysine to a deteriorated powder to the extent of rather less than half of the original lysine content of the fresh powder should restore the dietary deficiency of this amino-acid, although it may not raise the content of free lysine quite to its original level. The experiment in which 37° C. 60-day gas-stored H powder was supplemented with 1·25% of lysine equivalent to *c.* 47% of the original content of this acid, showed that the biological value was fully restored, and the protein efficiency greatly increased, although still below the level of the control, largely as a result of a reduced digestibility of the deteriorated protein (Part IV, Exp. 7). Lysine deficiency, therefore, appears to be the main biological defect of the proteins of gas-stored, deteriorated powders of high moisture content. This is in agreement with the conclusion reached from the chemical results that the protein-sugar reaction is largely responsible for other forms of deterioration in this powder.

In Fig. 1 the points for M powder at 37° C. (182 days, air) and H powder at 37° C. (60 days, 'extra air') lie off the curves in a direction which suggests a greater loss of nutritive value than would be expected from the fall in amino-nitrogen (lysine) alone. This would indicate that some additional factor has depressed the nutritive value of these powders. The biological tests (Part IV, Exp. 8) show that the addition of histidine, and possibly of arginine further improves the protein efficiency of deteriorated 'extra air' powder which has already been supplemented with lysine. The effect of supplementing with methionine was not investigated. The results obtained by the microbiological assay method (Part V) substantiate the conclusion that reduced availability of lysine is the major change undergone by the protein in the stored powders, and suggest loss of histidine, arginine and methionine as probable secondary factors. There is thus considerable evidence that in air-stored powder at least, and particularly when storage has been prolonged, changes occur in amino-acids other than lysine.

True digestibility does not appear to be so directly linked with the inactivation of lysine as the biological value, although it is obviously depressed in some way by storage, particularly at high temperatures. Support to this view is given by the behaviour of the M powder, the point for which lies badly off the true digestibility/amino-nitrogen curve (Fig. 1). Further work on this problem is in progress.

Protein deterioration on storage has previously been observed by chemical or biological methods in wheat and wheat flour (4), in soya beans (5, 6), in maize (5, 7), in egg white (8) and, since completion of the present work, in milk powder (9). Destruction of lysine by heat has been observed in milk (10), casein (11) and oat protein (12). With casein and oat protein some of the lysine was rendered unavailable to enzymic digestion, although it could still be released by acid (12, 13).

The analogy between these latter results on the inactivation of lysine by heat and those now reported for storage is striking. There is a discrepancy, however, in that 'pure' proteins such as casein, as well as foods containing both protein and carbohydrate, show inactivation of lysine by heat while, under the conditions of storage employed in the

present work on milk powder the presence of carbohydrate seems to be essential. Whether or not changes leading to the inactivation of lysine can occur in the absence of sugar under more severe storage conditions is not known, and requires investigation. A mechanism whereby the free ϵ-amino-groups of lysine might link up with other parts of the protein molecule, e.g. with free carboxyl groups, is obviously possible during prolonged storage as it is under the influence of heating at a high temperature for a short time.

The work described above forms part of a joint programme of the National Institute for Research in Dairying, the Food Investigation Board of the Department of Scientific and Industrial Research, and the Hannah Dairy Research Institute.

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APPENDIX

In view of the rapid deterioration in the nutritive value of the proteins which occurred on storage of dried skim milk of high moisture content (cf. Part IV, p. 347) the conditions were further defined under which milk of medium moisture content could be kept for long periods without injury to its proteins.

Samples of M powder (5% moisture) were, therefore, examined after storage for 26½ months at 28·5° C. in air and in nitrogen, and at 20° C. in air. No changes in biological value were observed, while the slight lowering in true digestibility from 93·6 to 91·9 and 92·0 for gas- and air-pack stored at 28·5° C., and to 92·5 for air-pack stored at 20° C. was without statistical significance. It has already been shown (Part IV, Exp. 5) that storage of this milk at 37° C. for 6 months caused a slight but significant decrease in the true digestibility but that no lowering of the biological value of the proteins occurred.

Chemical changes in the powders stored for 26½ months were, in general, very small, the free amino-nitrogen content showing a slight fall consistent with the values given in Fig. 17, Part III and Fig. 1, Part VI.

It follows that powders containing not more than 5% moisture can be stored in air- or gas-pack at temperatures up to 28·5° C. (83° F.) for at least 2 years without suffering detectable loss in the nutritive value of their proteins, though with a serious loss of flavour in the former and a less-marked loss in the latter (cf. Figs. 1 and 2, Part III). Furthermore, it seems likely that under all conditions of storage skim-milk powder will become unpalatable before it suffers any appreciable loss in the nutritive value of its proteins.

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369. DETERMINATION OF THE FREE AMINO-NITROGEN OF CASEIN AND OF FRESH AND DETERIORATED MILK PROTEIN BY THE VAN SLYKE METHOD

By C. H. LEA, *Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge and Department of Scientific and Industrial Research*

(With 1 Figure)

The manometric method of Van Slyke (1) which measures the nitrogen evolved on reaction of a free amino group with nitrous acid has long been known as a rapid and sensitive method for the estimation of amino acids. The α-amino-groups of the amino acids react quantitatively within about 5 min., while amino-groups in most other substances react considerably more slowly. Cystine, glycine and glycyl peptides give abnormally high values (1, 2). Levene & Van Slyke (3) found that the amount of gas given off by glycine increased slowly with time, so that after 30 min. the yield was 112% of theoretical; Kendricke & Hanke (2) found an even larger error. With glycyl-glycine as much as 135% of theoretical was obtained after 60 min. (1); glutathione (4) also gives high results. Schmidt (5) showed that tryptophane, which gave a theoretical result in 4 min., gave values of 110% after 30 min. and 116% after 60 min. The ε-amino-group of lysine reacts considerably more slowly than α-amino-groups, 15–20 min. at 20–24° C. being required for complete reaction (1, 6, 7). Deamination of the free ε-amino-group in lysine-containing peptides, however, proceeds approximately twice as rapidly as in lysine itself (8).

Comparatively little is known concerning the reaction between nitrous acid and proteins. Van Slyke & Birchard (9) found for a number of proteins that the free amino nitrogen corresponded approximately to one-half of the lysine nitrogen, and suggested that the free amino-groups of proteins are provided almost exclusively by the ε-amino-groups of lysine. In some proteins, however, the number of free amino-groups is obviously greater than can be accounted for by the ε-groups of lysine, and Sanger (10) has shown that in the rather exceptional case of insulin a relatively low content of lysine, and a multilayer structure with consequently increased exposure of terminal α-amino-groups (some of them of glycine), combine to reduce the contribution of the ε-groups of lysine to rather less than one-third of the total Van Slyke nitrogen.

Van Slyke & Birchard (9) found that the reaction between proteins and nitrous acid under the conditions of the determination reached a constant value after about 20–30 min. at 22–23° C., the figure then obtained for the free amino-nitrogen content of casein, as proportion of the total nitrogen, being 5·51%. Dunn & Schmidt (7) also found that the reaction with casein had reached completion in about 28 min. at 20° C. On the other hand, Kekwick & Cannan (11), working with egg albumin, observed a rapid initial reaction followed by a slower increase at an approximately linear rate, which proceeded for at least 100 min. at 21–23° C., and which they attributed to unspecified secondary reactions. Cannan, Palmer & Kibrick (12) observed a somewhat similar behaviour with β-lactoglobulin, but the secondary reaction was, in this case, very slow. Lieben & Loo (13), working with casein, observed a marked secondary reaction which, however, proceeded

at a diminishing rather than a uniform rate. By means of a correction based on observations taken after reaction for 30, 60 and 90 min., they attempted to deduce the true free amino-nitrogen, and hence the lysine content of the protein. The value obtained (5·8% on a weight basis) was far below that now accepted for the lysine content of casein.

In view of these conflicting results it was thought desirable to reinvestigate the effect of time of reaction on the apparent free amino-nitrogen content of casein, and to obtain this information also for the protein fractions from fresh milk and from deteriorated milk powder before proceeding to the routine estimation of changes in free amino-nitrogen occurring during the storage of experimental milk powders (14).

MATERIALS

Casein. Isoelectric casein was prepared from unheated fresh milk by the method of Cohn & Hendry (15), which avoids exposure to conditions more alkaline that pH 6·3 and contact with organic solvents. When required for use the casein was soaked in water at 0° C. overnight, brought into solution by the addition of 0·125 g. sodium carbonate per g. and made up to volume.

Milk protein. Unheated fresh milk was separated, dialysed in the presence of a little toluene at 0° C. for 7 days, freeze-dried, sieved and mixed. The product, which consisted of light, white glistening flakes, was stored at −20° C. and redispersed in water as required. Alternatively, if intended for use within a very few days, the dialysed protein suspension was held at −20° C. in sealed containers and thawed out as required.

Deteriorated milk protein. Spray-dried separated milk powder containing 7·6% of moisture was stored at 37° C. in an atmosphere of nitrogen for 60 days. The deteriorated powder, which was light brown in colour and of extremely poor solubility and flavour, was suspended in water, dialysed at 0° C. for 5 days, and freeze-dried. 100 mg. of the product, which dissolved quite readily in the acetic acid in the reaction chamber, were used for each determination.

METHOD

For investigation of the effect of period of reaction the protein suspension (4 ml.), or solid protein and water, was washed into the reaction chamber with glacial acetic acid (1 ml.) and water (1 ml.), deaerated for 2 min., 44% sodium nitrite (2 ml.) added, and the shaker and stopwatch started. The temperature of the room and of the water jacket was maintained accurately at 20° C. The reaction chamber was protected from light in order to avoid possible error from this cause (16).

Protein or partly deaminized protein is thrown out of solution as soon as admixture with the nitrous acid reagent occurs, and the reaction takes place in a heterogeneous system. A reaction chamber equipped with 2 ml. and 50 ml. bulbs only was, therefore, employed in order to minimize precipitation of protein above the level bathed by the liquid. In earlier experiments the vessel was shaken continuously throughout the determination, but this procedure was found to be of doubtful advantage and was replaced by shaking vigorously for 15 sec. immediately after mixing the reagents and subsequently at intervals of 15 min.

Defoaming agent. Diphenyl ether proved quite inadequate as defoaming agent in the presence of protein, and particularly of the protein-sugar complex which has marked foaming properties. It was necessary therefore to use secondary octyl (capryl) alcohol, despite the fact that this substance considerably augmented the '*c* correction', and that

this correction increased appreciably with the duration of the run. Commercial samples of capryl alcohol varied greatly in purity. The use of one drop of a redistilled fraction boiling within 1° C. of the correct figure was standardized. So far as could be ascertained by experiments with alanine extending over a period of 90 min. no error was introduced by the use of capryl alcohol, such as has been observed with defoaming agents containing amyl alcohol (17). A test carried out with some difficulty on a dialysed deteriorated milk powder for a reaction time of 15 min. without the use of capryl alcohol also indicated that no error resulted from use of the defoaming agent. Determinations of longer duration without capryl alcohol were not practicable.

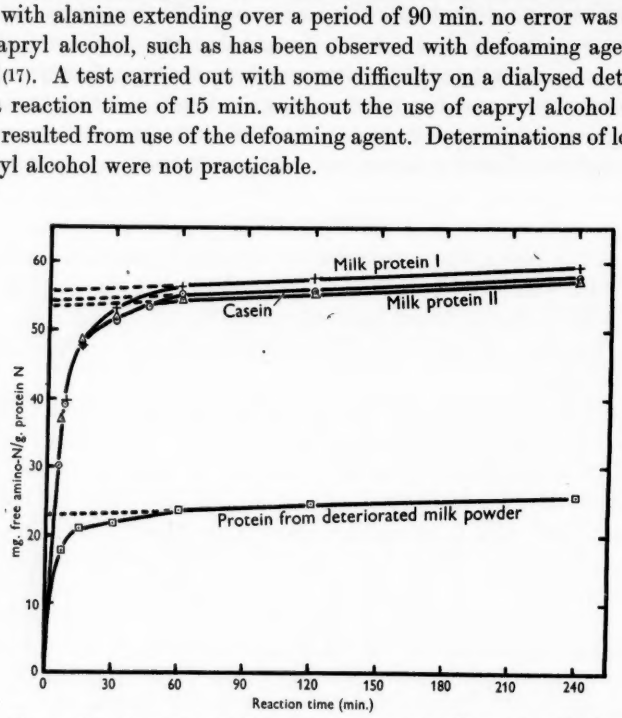

Fig. 1. The reaction of casein and of fresh and deteriorated milk protein with nitrous acid.

Effect of time of reaction

The results, which have been plotted in Fig. 1, show that the evolution of nitrogen proceeds at a very rapid rate during the first 5 min., but that the rate then falls off and in no case is the reaction complete within 30 min. at 20° C. The reaction/time curves for casein and for fresh and deteriorated milk protein, however, all merge after about 60 min. into a very slow and approximately linear rate of increase of very nearly 1 unit per hr., which can probably be attributed to some 'secondary' process such as a reaction between nitrous acid and amide or other non-amino groups, or to a very slow hydrolysis of the peptide chain with liberation of further free amino groups.

The effect of this secondary reaction can best be eliminated by extrapolation of the linear portion of the curve back to zero time, or, under the conditions employed in these determinations, by subtracting 1 unit from the 60 min. value. For the routine examination of deteriorated milk powders (14), however, a more rapid method was desirable and a reaction time of 30 min. was standardized, the values so obtained being corrected by the addition of 2 units. Since some samples of milk protein appear to reach the linear portion of the reaction/time curve appreciably earlier than others an error of as much as ± 1 unit may be introduced by this approximation.

Nature of the groups reacting

Free α-amino-groups at the end of protein chains or sub-units can be expected to react, together with part of the ε-amino-groups of lysine, during the first 5 min. The decreasing evolution of gas between 5 and 60 min. presumably includes completion of the reaction with lysine (some residues of which may perhaps be less 'accessible' than others), together with any artifact introduced by slow penetration of the reagent into the interior of solid particles, and the possible effect of continued reaction of a few terminal free α-amino-groups of acids which give anomalous results in the Van Slyke determination over long periods.

Since, in the case of lysine itself, the ε-amino-group is known to have a much stronger tendency to associate with formaldehyde than has the α-amino-group (18) it would not be unreasonable to suppose that in the protein the ε-amino-groups of the lysine side chains react with aldose more readily than do the few terminal α-amino-groups. On this view the deteriorated protein might still contain most of the few terminal free α-amino-groups originally present.

The reaction/time curve for the deteriorated protein showed a distinct tendency for the reaction between 15 and 60 min. to proceed at a nearly uniform instead of at a steadily diminishing rate. In this case the situation is complicated by the possibility of the liberation of amino-groups from the union, probably with lactose, into which they have entered during storage of the powder. There is no sign, however, of such a reaction proceeding to any considerable extent.

Discussion

When adequate precautions are taken Van Slyke determinations on intact protein yield quite reproducible results. Much of the more recent data in the literature has been accumulated for β-lactoglobulin. When the values obtained by Miller (30 min. (19)), Cannan *et al.* (30 min. (12)) and Brand, Saidel, Goldwater, Kassel & Ryan (reaction time unspecified (20)) are corrected to the same molecular weight of 42,000, they all correspond approximately to the presence of 37 free amino-groups per molecule, of which 33 can be accounted for by the ε-amino-groups of the lysine present (20). Sanger's elucidation of the relationship between the free amino content and the structure of insulin has already been referred to (10).

The value obtained for casein in the present work (54·0 mg. free amino-nitrogen/g. total nitrogen) is in fair agreement with the early value of Van Slyke and Birchard (55·1). Of this total the ε-amino-groups of lysine, assumed to constitute 8·3% on a weight basis or 10·1% on a nitrogen basis of the casein, will account for 50·5 mg. or 93–94%, leaving only 6–7% to be accounted for by the free α-amino-groups exposed at the ends of protein chains or sub units. Even this estimate may be too high in view of the known tendency of the Van Slyke method to give too high values with certain free amino-acids, particularly after prolonged reaction.

The free amino-content of milk protein obviously varies appreciably with the sample of milk. The two samples tested (Fig. 1) gave values of 53·3 and 55·5. Protein from two fresh spray-dried powders gave 55·5 and 59·8. Such variation is probably inevitable in view of the known variation in the composition of the protein of milk, particularly in relation to stage of lactation (21).

SUMMARY

The reaction of casein and of fresh and deteriorated milk protein with nitrous acid has been followed at constant temperature for 4 hr. in the manometric apparatus of Van Slyke, and simplified procedures suggested whereby the method can be utilized for investigation of the deterioration of the protein of separated milk powder during storage.

Technical assistance in this work was given by Mr L. J. Parr. The work was carried out as part of the programme of the Food Investigation Board of the Department of Scientific and Industrial Research.

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370. THE REACTION BETWEEN MILK PROTEIN AND REDUCING SUGAR IN THE 'DRY' STATE

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(With 1 Figure)

It has been shown that a progressive loss of free amino-nitrogen and an approximately corresponding increase in combined sugar occur in the undialysable (protein) fraction of separated milk powder of high moisture content during storage (1). These observations suggested that a reaction of the type described by Maillard (2) between amino-acids and reducing sugars was responsible for part at least of the undesirable changes in flavour, colour and solubility which develop in milk powder under such conditions. It was still possible, however, that denaturation of the protein, involving changes in molecular configuration with exposure of sulphydryl and other groups such as is known to occur in heat denaturation, was primarily responsible for the observed deterioration in solubility, etc., the combination with sugar being a side reaction of relatively minor importance. In order to test this point, it was necessary to store milk protein in the presence and absence of sugar. Opportunity was taken to compare, at the same time, the effect of lactose at the concentration normal to milk with that of glucose at the concentrations at which its presence has been reported but not fully confirmed in milk (3, 4), and with that of sucrose.

Exp. 1

Unheated, fresh milk was separated and dialysed at 0° C. in the presence of a little toluene against repeated changes of distilled water for a total period of 7 days. The solids content of the dialysed milk was determined and the requisite quantities of the various sugars added to portions of the fluid, which were then freeze-dried in separate containers in the same apparatus. α-Lactose hydrate was converted to the equilibrium mixture of α- and β-forms before incorporation in the milk. The pH of the fluids ranged between 6·95 and 7·00. The dried products, of which the compositions are given in Table 1, were sieved, mixed and allowed to equilibrate for 7 days at 20° C. over dilute sulphuric acid to the required moisture content. The material was then transferred to a number of small open vessels each containing the equivalent of 2 g. of protein, and stored over sulphuric acid producing a relative humidity of 55%. After suitable intervals samples were withdrawn and examined for free amino and total nitrogen content, for solubility of the protein and for colour.

For the determination of solubility a portion of the sample containing 0·5 g. of protein was reconstituted by creaming with a little water, diluted until a total of 12·5 g. of water had been added and held at 0° C. overnight. The fluid was then maintained at 20° C. for 20 min., shaken vigorously in a mechanical shaker for 3 min. and centrifuged at 3000 r.p.m. for 15 min. Total solids and nitrogen content were determined on separate aliquots of the supernatant fluid, and the percentage of soluble nitrogen calculated. Error in estimating the solids content of the supernatant fluid affects solubility as determined by the nitrogen method less seriously than when a direct weight method is used.

Free amino-nitrogen was determined in the stored samples by the Van Slyke method (5) without previous dialysis, and the values expressed as mg. per g. of the total, i.e. protein, nitrogen. Colour was measured as previously described (1).

Results. The results which are plotted in Fig. 1 show that there was no loss of free amino-nitrogen, no appreciable loss of solubility, and no discoloration when dialysed milk protein was stored alone at 37° C. and 55% relative humidity for as long as 90 days. After 150 days, however, solubility had fallen seriously, although free amino-nitrogen and colour remained unchanged.

The incorporation of a high concentration of the non-reducing sugar sucrose did not cause any deterioration in the protein, in fact it exerted a marked stabilizing influence, the protein admixed with sucrose being still fully soluble after 150 days at 37° C., when the experiment ended.

The addition of reducing sugar to the dialysed protein caused a loss of free amino-nitrogen on storage, a loss which the initial values show had commenced even during the preliminary equilibration at 20° C. At 37° C. the free amino-content of the protein fell rapidly at first and then more slowly, the order being strong (11·1%) glucose, lactose (144%), weak (1·9%) glucose. Changes in solubility were similar in that all three samples containing added reducing sugar also showed loss of solubility, but differed in that all samples showed an induction period of about 10–20 days during which solubility remained unaffected while free amino-content fell rapidly. This behaviour has previously been observed with milk powder (1). The relative order of the samples was also different, the weak glucose sample becoming insoluble much more rapidly than that containing lactose, despite a difference in sugar concentration of the order of 1 : 76 in favour of the lactose.

Only in the presence of reducing sugar did discoloration develop on storage, glucose being much more objectionable in this respect than lactose.

Exp. 2

The extraordinary efficiency of glucose in causing loss of solubility and discoloration, as compared with lactose, suggested that the small and very variable quantities of glucose which have been reported in cows' milk might exert a considerable influence on the keeping properties of the powder. Further investigation, however, was necessary to determine the behaviour of a small concentration of glucose in the presence of a much greater concentration of lactose, as would be the case in milk. The method used was as before, except in that the protein-sugar solutions (pH 7·0–7·05) were frozen more rapidly at −20° C. prior to drying in order to reduce the risk of separation of the components of the mixture during freezing. Equilibration at 20° C. prior to storage at 37° C. was omitted. The compositions of the materials stored are given in Table 1, and the changes in free amino-nitrogen, in solubility and in colour in Fig. 1.

Results. The results of the second experiment confirm those of the first in showing the rapid reaction of a small quantity of added glucose with free amino-groups of the protein, combination in this case reaching its maximum within 10 days. Lactose reacted more slowly but, being present in much greater concentration, the total amount of amino-group blocked was greater, amounting to over half of the original free amino-nitrogen content after 40 days. The reaction then became very much slower, despite the presence of unreacted free amino-nitrogen and of a large excess of lactose (cf. p. 373). Dilution of

the protein-glucose mixture with a large volume of sucrose did not prevent or even slow down reaction between the glucose and the amino-groups. The loss of amino-nitrogen in the presence of lactose + glucose, however, closely followed the lactose curve (Fig. 1), indicating that the great excess of lactose present was successfully competing with glucose for the protein amino-groups and largely preventing the glucose-protein reaction.

Table 1. *Composition of the milk protein-sugar mixtures stored*

Exp.	Sample	Sugar added (g./100 g. dialysed separated milk solids)	g./100 g. total dry solids: Dialysed separated milk solids	g./100 g. total dry solids: Added sugar	Approx. sugar content on whole milk basis (%)
1	Control	—	100·0	—	—
	Weak glucose	1·9 glucose	98·1	1·9 glucose	0·06 glucose
	Strong glucose	11·1 glucose	90·0	10·0 glucose	0·37 glucose
	Lactose	144·0 lactose	41·0	59·0 lactose	4·8 lactose
	Sucrose	144·0 sucrose	41·0	59·0 sucrose	4·8 sucrose
2	Control	—	100·0	—	—
	Weak glucose	1·9 glucose	98·1	1·9 glucose	0·06 glucose
	Lactose	144·0 lactose	41·0	59·0 lactose	4·8 lactose
	Sucrose	144·0 sucrose	41·0	59·0 sucrose	4·8 sucrose
	Glucose + lactose	1·9 glucose, 144·0 lactose	40·7	58·5 lactose, 0·8 glucose	0·06 glucose, 4·8 lactose
	Glucose + sucrose	1·9 glucose, 144·0 sucrose	40·7	58·5 sucrose, 0·8 glucose	0·06 glucose, 4·8 sucrose

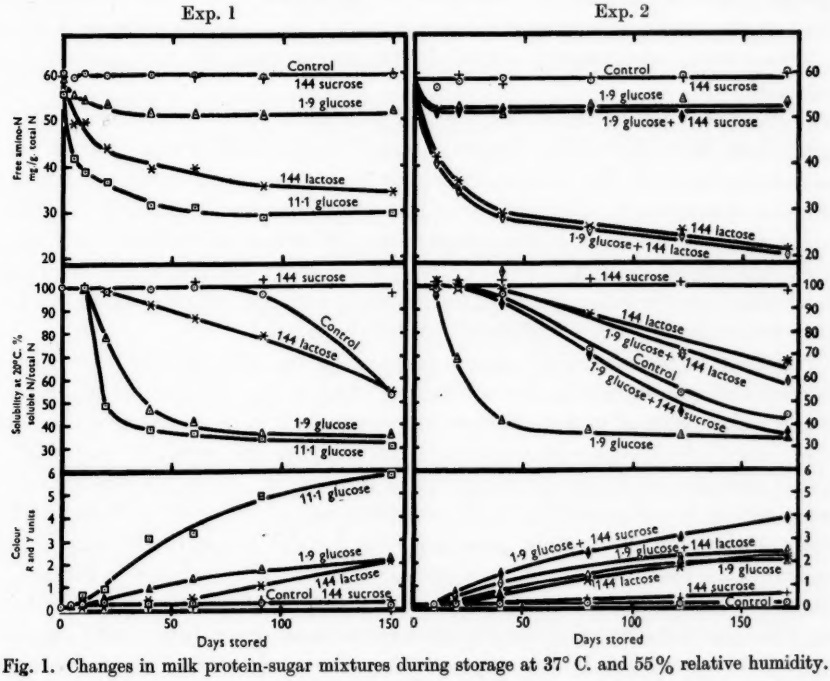

Fig. 1. Changes in milk protein-sugar mixtures during storage at 37° C. and 55% relative humidity.

Loss of solubility in the presence of reducing sugar again showed a sharp induction period of the order of 10–20 days, during which free amino-nitrogen disappeared rapidly without causing any change in solubility. The very serious effect on solubility of a small concentration of glucose, when present as the only sugar, was again apparent. Dilution of the glucose with a large quantity of sucrose strikingly delayed the development of

insolubility, and lactose was even more efficient than sucrose in this respect (Fig. 1). Again the glucose + lactose and lactose alone curves were almost identical, indicating practically complete suppression of the glucose reaction by the large excess of lactose. The control protein, without added sugar, became insoluble much earlier in this than in the previous experiment, but the addition of a high concentration of sucrose was again completely effective in preventing loss of solubility.

Neither the control protein nor the protein plus sucrose became discoloured on storage. Weak glucose, strong lactose or both together produced practically the same degree of discoloration, again suggesting almost complete suppression of the glucose reaction by lactose. Rather surprisingly the sample containing glucose + sucrose became even darker, indicating that sucrose is not inert from the view-point of discoloration when reducing sugar is also present.

Discussion

The Van Slyke data show that the reducing sugars glucose and lactose react with the free amino-groups of milk protein when sugar and protein are stored together at 37° C. and 55% relative humidity, glucose being the more reactive of the two, but that sucrose does not react with protein in this way. Only those samples in which combination between reducing sugar and protein amino-groups occurred became discoloured on storage, the degree of discoloration produced depending on the nature of the sugar participating as well as on the number of sugar molecules and protein amino-groups reacting. Apparently the glucose-amino compound is degraded more readily than the corresponding lactose compound.

The effect of sugars on the development of insolubility of the protein is complex, and has not been fully elucidated. Results obtained to date suggest that at least two mechanisms are involved. The potential aldehyde group of reducing sugars combining with free amino-groups tends to destabilize the protein and produce insolubility. Whether this is achieved by acceleration of the normal process of denaturation (itself far from completely understood), or is the result of a bridging or other chemical action involving the sugar molecule and reactive groups in the protein is not clear. Glucose shows this type of action predominantly and its influence is definitely harmful.

In addition to this action the sugars, particularly the disaccharides when present in relatively high concentration, show a retarding effect on the development of insolubility, whether aldose induced or not. Lactose appears to be even more effective than sucrose in combating glucose-induced insolubility, probably as the result of successful competition with glucose for the amino-groups of the protein, but in the absence of added glucose lactose is less beneficial than sucrose, presumably because of the harmful effect of its own aldehyde group. Lactose, possessing both destabilizing and stabilizing properties, accelerated the onset of insolubility in one experiment and retarded it in another. It is of interest to compare these results with the observations of Bate-Smith & Hawthorne (6) and of Brooks & Hawthorne (7) on the effect of the same sugars in the dried egg system.

Insolubility developed in the absence and presence of reducing sugar showed some points of dissimilarity; the pure protein precipitate, for example, appeared to be finer and settled less readily under the influence of gravity. It has already been pointed out in the case of separated milk powder that loss of solubility in water at 60° C. occurs at a later stage of deterioration than does loss of solubility in water at 20° C., although the limiting values ultimately reached are the same (1). The artificial protein-sugar systems behaved

similarly. It is of interest to note, however, that control protein of depreciated solubility at 20° C. remained fully soluble at 60° C., whereas sugar-containing samples of similar solubility at 20° C. showed considerable losses at 60° C. (Table 2).

Table 2. *Comparison of solubilities in water at* 20° *and at* 60° C.

Sample	Sugar added (g./100 g. dialysed separated milk solids)	Solubility after days at 37° C. 122 20° C.	122 60° C.	170 20° C.	170 60° C.
Control	—	54	98	44	100
Sucrose	144·0 sucrose	102	102	97	103
Lactose	144·0 lactose	70	99	67	82
Glucose + lactose	1·9 glucose, 144·0 lactose	70	96	59	73
Glucose + sucrose	1·9 glucose, 144·0 sucrose	46	80	37	53
Glucose	1·9 glucose	34	34	34	30

The nature of the protein-sugar complex

The reaction between 'weak' (1·9%) glucose and protein ceased when some 70% (Exp. 2) or 80% (Exp. 1) of the theoretical equivalent of amino-nitrogen had been inactivated, despite the fact that amino-group equivalent to six times the amount of glucose added was available. When glucose (11·1%) roughly equivalent to all the amino-group present was added, reaction ceased when only a little over 50% of the free amino-nitrogen had been inactivated. At the other extreme, when lactose equivalent to over six times the total free amino-nitrogen was present, as in milk, only some 45% (Exp. 1) or 65% (Exp. 2) of the amino-nitrogen had been inactivated at the end of the experiment, although the reaction was still proceeding very slowly. The higher of these two figures is in good agreement with the data obtained during the storage of milk powder (1); the lower may be due to partial separation of the constituents of the mixture during slow freezing prior to drying. In all cases a likely explanation of the failure of the minor reactant to combine completely despite the presence of a large excess of the other would appear to lie in the difficulty of access of the reactive groupings to one another in the solid system. Difficulty of access of the relatively large (as compared with nitrous acid) sugar molecules to certain of the free amino-groups of the protein molecule, due to folding may however be an important factor.

It has been pointed out elsewhere (8) that the distinction between the amino-sugar reaction proper and caramelization is vague, and that one molecule of amino-compound can, under certain conditions, cause the degradation of as many as ten, and perhaps more, molecules of sugar. Amino-groups can therefore be considered as capable of catalysing the caramelization of sugar. Since caramelized sugar is strongly adsorbed by protein (9, 10) a definite possibility existed that the experimental results obtained in the present and previous (1) work might be due to the blocking of protein amino-groups by some chemical reaction other than with sugar (e.g. with protein carboxyl groups), and that caramelized sugar adsorbed on the protein was simulating chemically combined sugar. The fact that in the present experiments considerable amounts of bound sugar have been found in samples of dialysed milk protein which were only slightly discoloured suggests that caramelization followed by adsorption can hardly be the mechanism by which sugar first becomes attached to protein during the storage of 'dry' protein-sugar mixtures. Further evidence, however, was sought on this point.

Relation between the number of protein amino-groups reacting and the amount of sugar bound. The dialysed protein fraction from separated fresh milk was admixed with a known amount of glucose or of lactose hydrate (*c.* 1 g./g. protein nitrogen), frozen rapidly, freeze-dried and stored at 37° C. and 55% relative humidity for 31 or 116 days. The amount of free (dialysable) sugar was then determined by reconstitution and dialysis to equilibrium at 0° C. against an equal volume of water, using the cuprometric method of Somogyi (11), and the bound sugar estimated by difference. The free amino-nitrogen content of the protein was simultaneously determined. The results (Table 3) show that

Table 3. *Relation between loss in free amino-nitrogen and increase in undialysable sugar during the storage of protein-sugar mixtures*

		Loss of amino-nitrogen		Increase in undialysable sugar (as glucose or lactose)	
Material	Days stored at 37° C. and 55% R.H.	(mg. N/g. total N)	Calculated as glucose or lactose (mg./g. total N)	As found by equilibrium dialysis method	As found by direct titration after complete dialysis
Milk protein + glucose	31	34·0	437	409 (94%)	395 (89%)
Milk protein + lactose	116	18·1	443	437 (99%)	397 (90%)

for every free amino-group which disappeared roughly one molecule of glucose or of lactose became undialysable. When the bound sugar content of the protein was determined after complete dialysis by direct titration in presence of the protein a figure corresponding to about 90% of the loss of free amino-nitrogen was obtained. This value is in agreement with the figure of 88% obtained by the same technique from experiments on the storage of experimental milk powders (1), and suggests that the observed deviation from 100% in the relationship between loss of free amino-nitrogen and increase in combined sugar was largely due to error in the direct method used for the estimation of combined sugar, and that one molecule of sugar does, in fact, react for each protein amino-group inactivated.

As with milk powder (1) the reduction in formol titration on storage was unable to account for more than a small fraction (in this case less than 20%) of the loss of amino-nitrogen found by the Van Slyke method, or of the increase in combined sugar.

Test for hydrolysis or dissociation of the protein-sugar complex during dialysis. Two samples of deteriorated milk powder and one of a stored milk protein-glucose mixture were allowed to dialyse, in the presence of a little toluene, against distilled water at 0° C. for periods in excess of the normal 5 days, free amino-nitrogen and sugar (by the direct titration method) being estimated at intervals. The initial free amino-nitrogen content of the fresh protein was of the order of 60.

The results for the milk powder samples (Table 4, Exps. 1 and 2) show that little if any loss of sugar occurred during continued dialysis between 3 and 20 days. The slight changes in free amino-nitrogen content of the protein noticeable in Exp. 3 do not greatly exceed the error of the determination and would, in any case, indicate slight hydrolysis of a protein-glucose compound (particularly during the first day of dialysis) rather than loss of adsorbed sugar. Tests for shorter periods of dialysis than 3 days could not be made with milk powder owing to the presence of appreciable quantities of non-protein amino-nitrogen. Bound sugar could not be estimated in either system until the excess of free sugar had been removed.

Table 4. *Effect of continued dialysis on the apparent free amino-nitrogen and bound sugar contents of deteriorated milk protein*

Exp. no.	Material	Stored at 55% R.H. for	Dialysed at 0° C. (days)	Free amino-N (mg./g. total N)	Apparent sugar (mg./g. total N)
1	Milk powder	60 days at 37° C. in N_2	5	23·7	880 (as lactose)
			10	23·7	860 (as lactose)
			20	25·0*	820 (as lactose)*
2	Milk powder	112 days at 28·5° C. in N_2	3	35·6	690 (as lactose)
			5	34·6	680 (as lactose)
			11	35·0	680 (as lactose)
			20	34·2	690 (as lactose)
3	Milk protein + glucose	9 days at 37° C. in air	0	31·5	—
			1	32·8	450 (as glucose)†
			2	32·7	410 (as glucose)
			4	32·9	410 (as glucose)
			8	34·2	390 (as glucose)
			16	34·0	390 (as glucose)

* Signs of bacterial decomposition; amino-nitrogen 49·2 and sample putrid after 40 days.

† Dialysis incomplete, free sugar still present.

Prolonged dialysis therefore failed to show a progressive loss of bound sugar without corresponding increase in free amino-nitrogen such as might have been expected had the sugar been loosely held by adsorption. While it is still possible that adsorbed sugar might have been sufficiently strongly held to resist such treatment, the balance of the evidence available certainly favours, as first stage in the deterioration process, a chemical reaction between protein amino-groups and reducing sugar, whereby one sugar residue becomes attached to the protein molecule for each free amino-group destroyed.

These observations on the nature of the protein-sugar complex must be regarded as of a preliminary nature; further work on the protein-sugar reaction and its influence on deterioration is in progress.

SUMMARY

'Dry', dialysed milk protein was stored for 6 months at 37° C. and 55% relative humidity alone and in the presence of small proportions of glucose, of high proportions of lactose and of sucrose, and of mixtures of these sugars.

The reducing sugars combined with free amino-groups of the protein, apparently in a 1 : 1 ratio; sucrose did not. The reaction did not proceed to completion, probably owing to difficulty of access of the reactive groups to one another. Only when the sugar-amino reaction occurred did discoloration ensue.

Glucose reacted more rapidly with the protein than did lactose, and the complex formed became discoloured and insoluble in both cold and hot water much more rapidly.

Sucrose and lactose both greatly delayed the onset of glucose-induced insolubility, lactose being the more efficient of the two. They did not prevent discoloration.

The protein alone became insoluble in cold but not in hot water after prolonged storage, but did not discolour. This change was prevented by sucrose. The behaviour of lactose was inconsistent, loss of solubility being accelerated in one experiment and retarded in another.

It is suggested that the effect of sugars on the development of insolubility in proteins includes (*a*) acceleration by reason of the reducing group-amino reaction, and (*b*) retardation under the influence of relatively high concentrations of carbohydrate, particularly

of disaccharide. Glucose behaves predominantly according to mechanism (*a*), sucrose entirely according to mechanism (*b*), and lactose according to both.

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371. THE PROPERTIES OF NEW ZEALAND BUTTERS AND BUTTERFATS

I. IODINE, REICHERT AND SAPONIFICATION VALUES AND SOFTENING POINTS OF MONTHLY SAMPLES OF BUTTERFATS FROM NINE COMMERCIAL FACTORIES OVER FOUR YEARS

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(With 6 Figures)

The variations in the chemical and physical properties of butterfat have already been extensively studied. In many European countries, e.g. Holland, Denmark, Ireland, where examination is made of random samples of butterfats from butters from every factory at intervals throughout the year as part of the regular 'Butter Control Service' to ensure the absence of adulteration with other fats, the information available on the variations in the properties is very complete. Detailed studies of the butterfats from some New Zealand butters have been made by English workers (1, 2, 3), but information has not hitherto been published on variations throughout the year. The present paper presents the results of analyses of butterfats from butters collected monthly from nine commercial factories selected as representative of the main butter-producing districts, which are located throughout the two main islands, from lat. 35° S. to lat. 46° S. The factories chosen were as follows:

No. 1	Kaitaia	(North Auckland)	No. 6	Awahuri	(Manawatu)
No. 2	Whangarei	(North Auckland)	No. 7	Golden Bay	(Nelson)
No. 3	Morrinsville	(Waikato)	No. 8	Ashburton	(Canterbury)
No. 4	Pio Pio	(King Country)	No. 9	Dunedin	(Otago)
No. 5	Stratford	(Taranaki)			

The samples forwarded were a 4 lb. sample from a single churning. Where the cream supply was such that it had to be distributed into more than one holding-vat, the sample from any one factory may not always have been representative of the whole factory output. It will be shown later that the variations from district to district were not large. Hence variations in the properties of the butterfat due to change in locality from which the samples were drawn within the factory supply area are not likely to be significant. The sampling was commenced in November 1938 and continued until October 1942.

Preparation of the samples of butterfat

The butters were placed in a 12 cm. glass funnel with rubber tubulure and screw-clip in a water-oven at 60–70° C. When the butter was melted the serum was drawn off, and the butterfat was passed twice through filter paper.

Methods of analysis

Iodine values, saponification values and Reichert values for the butterfats were determined by the British Standard Method (4). Softening points were measured by Barnicoat's modification of the falling-ball method (5).

Results

Periodicity of the changes in butterfat properties

The results obtained for iodine values, saponification values, Reichert values and softening points of the samples at monthly intervals for the 9 individual factories are plotted in Figs. 1, 2, 3 and 4. These graphs clearly show that the trend of variations of any one

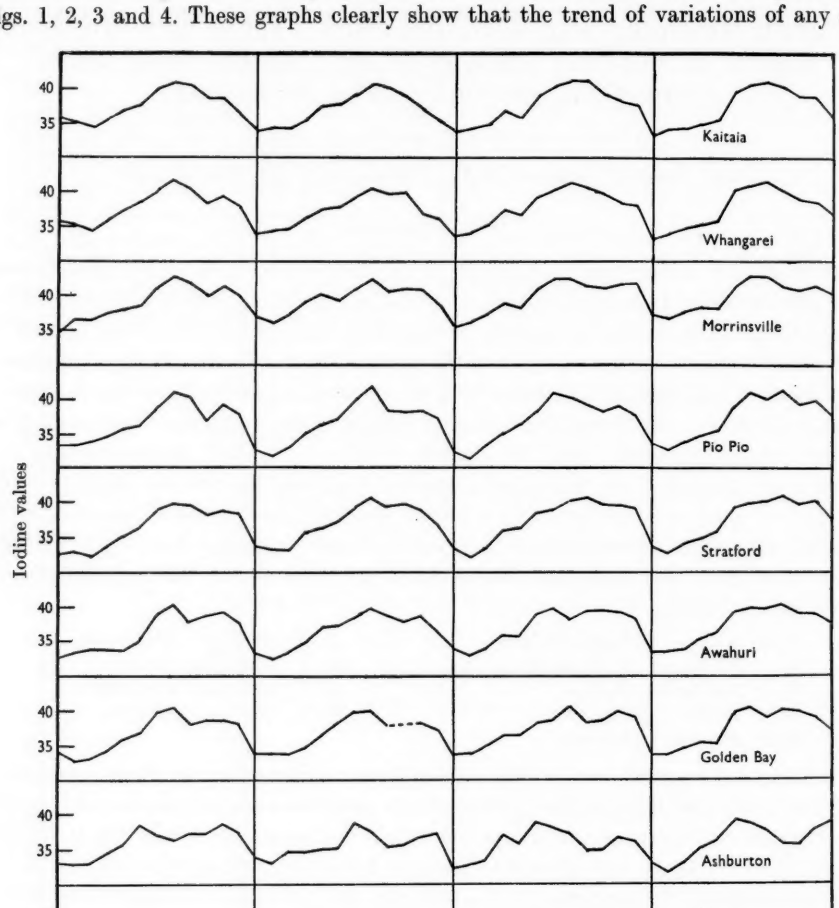

Fig. 1. Iodine values of monthly samples of butterfats from 9 factories over 4 years.

property throughout the season was remarkably uniform, both for different factories in the one season, and for any one factory in the four seasons. The maximum and the minimum values for any one property occurred at approximately the same time in every factory, but the time at which these maxima and minima occurred was not the same for all of the four properties studied. This is evident from Table 1 which shows the month of

the year in which the maximum and minimum results were obtained in each year. The results may be summarized as follows:

Iodine value	Minimum November to January	Maximum April to October
Reichert value	Minimum February to June	Maximum August to October
Saponification value	Minimum April to June	Maximum October to January
Softening point	Minimum August to November	Maximum December to June

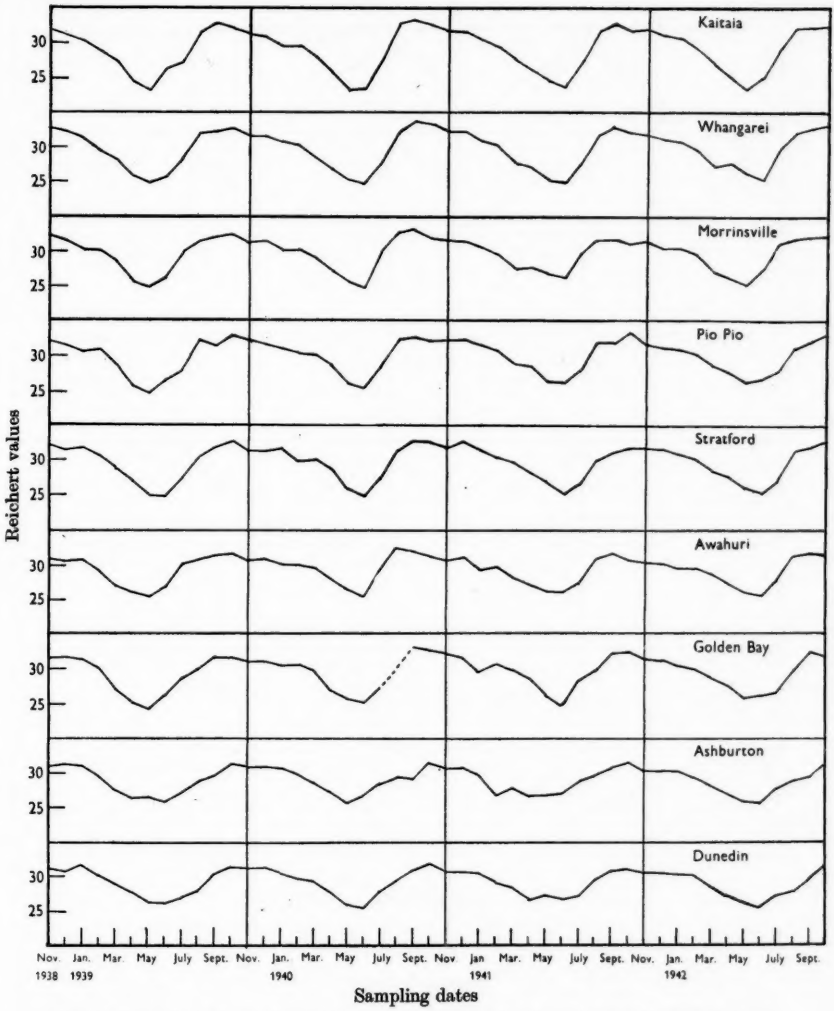

Fig. 2. Reichert values of monthly samples of butterfats from 9 factories over 4 years.

Since dairying for butter manufacture is markedly seasonal in New Zealand, the cows calving in the spring months (July–September) and, for the most part, completing lactation in May and June, periodicity could be attributed either to a regular lactational trend or to a regular seasonal change in the nature of the feed supply. Evidence to differentiate between these two factors will form the subject of a later paper from this

Institute. It may be pointed out, however, that the minimum iodine value in New Zealand occurs in the summer months, i.e. at the stage in the season when the maximum values are reported in Northern-hemisphere countries.

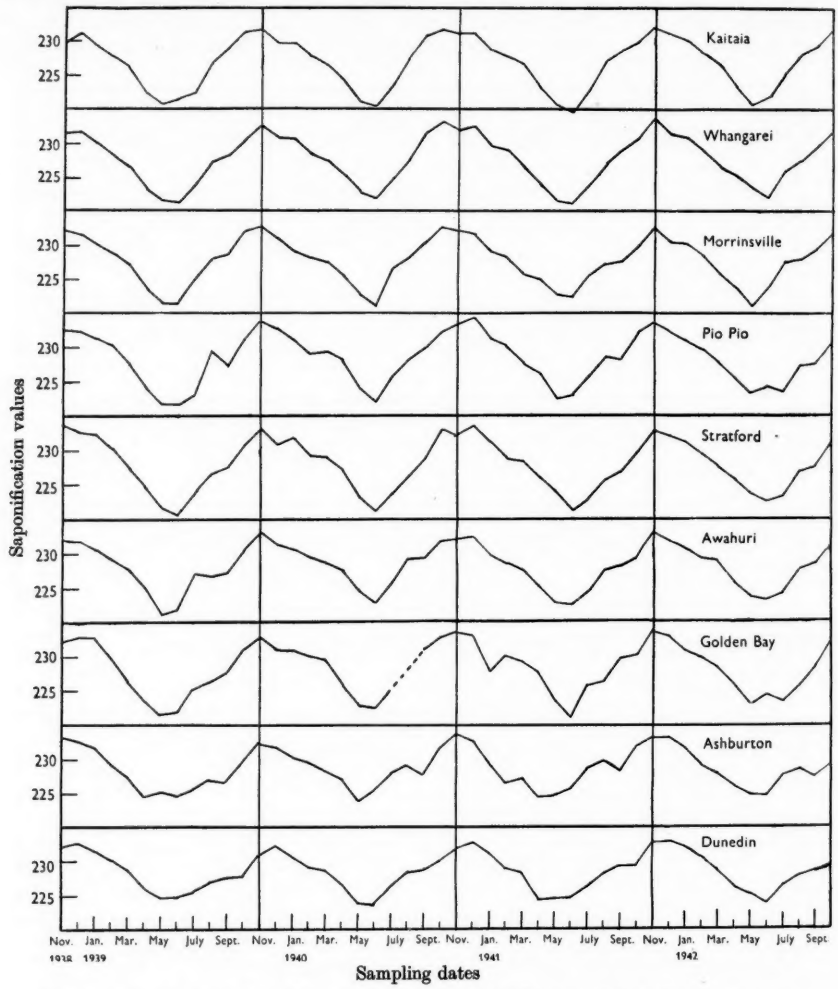

Fig. 3. Saponification values of monthly samples of butterfats from 9 factories over 4 years.

Average results

Since the results show similar trends of variation over each of the 4 years under review it is permissible to assume that there were no very unusual factors influencing the properties of the butterfat during the period and the averaged results may be taken as representing the normal trend of the change in properties of New Zealand butterfat throughout the year. The weighted averages have been calculated rather than the straight average of the results obtained, since the greater proportion of the butter production in New Zealand is from the North Island factories, and the quantities produced per month

vary widely throughout the season. McDowall (6) has cited the average distribution of the butter production of New Zealand factories over the 2 years 1943–5, and has expressed the production for each month as a percentage of the total yearly production, for both the North Island and the South Island districts. In the present calculations it has been assumed that the same monthly distribution applied over the years 1938–42.

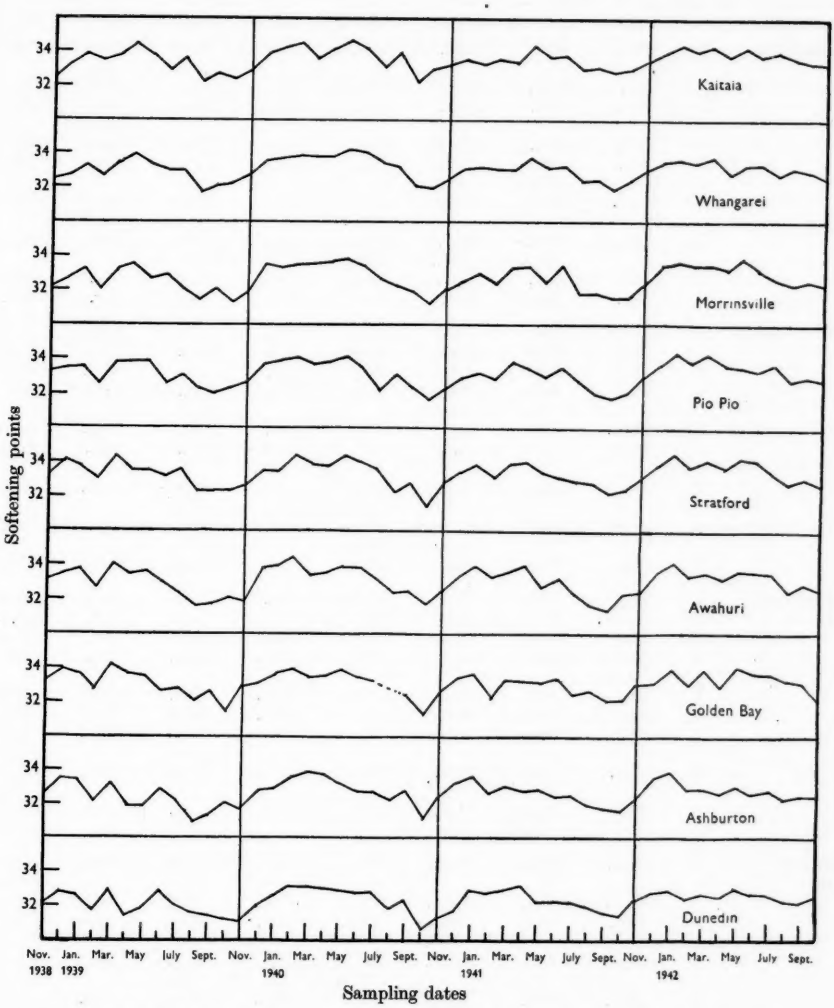

Fig. 4. Softening points of monthly samples of butterfats from 9 factories over 4 years.

The weighted average results for the four properties of butterfat studied are given in Table 2, and are plotted in Fig. 5, as from July to the following June, corresponding fairly closely to the lactational year.

Table 1. *Months of year in which maximum and minimum results for iodine values, Reichert values, saponification values and softening points were obtained over 4 years*

Property of butterfat	Factory no.	Months of year in which *minimum* values were obtained				Months of year in which *maximum* values were obtained			
		1938–9	1939–40	1940–1	1941–2	1938–9	1939–40	1940–1	1941–2
Iodine value	1	Jan.	Nov.	Nov.	Nov.	June	June	June–July	June
	2	Jan.	Nov.	Nov.	Nov.	June	June	June	June
	3	Nov.	Dec.	Nov.	Dec.	June	June	May–June	May
	4	Dec.	Dec.	Dec.	Dec.	June	June	May	July
	5	Jan.	Dec.–Jan.	Dec.	Dec.	June	June	July	July
	6	Nov.	Dec.	Dec.	Nov.–Dec.	June	June	May	July
	7	Dec.	Nov.–Jan.	Nov.–Dec.	Nov.–Dec.	June	June	June	May
	8	Dec.	Dec.	Nov.	Dec.	Sept.	May	Apr.	Oct.
	9	Jan.	Jan.	Jan.	Jan.	Oct.	Oct.	Apr.	Apr.
Reichert value	1	May	May	June	May	Oct.	Sept.	Sept.	Sept.
	2	May	June	June	June	Oct.	Oct.	Sept.	Sept.
	3	May	June	June	May	Oct.	Oct.	Sept.	Sept.
	4	May	June	May–June	May	Oct.	Oct.	Sept.	Oct.
	5	May	June	June	June	Oct.	Oct.	Sept.	Nov.
	6	May	June	June	June	Sept.–Oct.	Oct.	Aug.	Sept.
	7	May	June	June	May	Sept.	Sept.–Oct.	Sept.	Oct.
	8	June	May	Feb., Apr., May	May–June	Oct.	Oct.	Oct.	Oct.
	9	June	June	June	June	Oct.	Oct.	Oct.	Oct.
Saponification value	1	May	June	June	May	Oct.	Nov.	Oct.	Nov.
	2	June	June	June	June	Oct.	Nov.	Oct.	Nov.
	3	May	June	June	May	Nov.	Nov.	Oct.	Nov.
	4	May–June	June	May	May	Nov.	Nov.	Dec.	Nov.
	5	June	June	June	June	Nov.	Nov.	Dec.	Nov.
	6	May	June	June	June	Nov.	Nov.	Dec.	Nov.
	7	May	June	June	May	Dec.–Jan.	Nov.	Nov.	Nov.
	8	Apr.	May	Apr.	June	Nov.	Nov.	Nov.	Nov.–Dec.
	9	May	May–June	Apr.	June	Dec.	Dec.	Dec.	Dec.
Softening point	1	Aug.	Sept.	Sept.	Nov.	Apr.	May	Apr.	Jan.–Mar.
	2	Aug.	Oct.	Sept.	Nov.	Apr.	May	Apr.	Mar.
	3	Oct.	Oct.	Sept.–Oct.	Nov.	Apr.	May	June	May
	4	Sept.	Oct.	Sept.	Aug.–Oct.	May	Feb.–May	Mar.	Jan.–Mar.
	5	Aug.	Oct.	Sept.	Oct.	Mar.	Feb.	Apr.	Jan.
	6	Aug.	Oct.	Sept.	Aug.	Mar.	Feb.	Apr.	Jan.
	7	Oct.	Oct.	Sept.	Oct.	Mar.	Feb.	Jan.	May
	8	Aug.	Oct.	Oct.	Aug.	Dec.	Mar.	Jan.	Jan.
	9	Nov.	Oct.	Oct.	Sept.	Dec.–Mar.	Feb.	Apr.	May

Table 2. *Weighted monthly average iodine values, Reichert values, saponification values and softening points of butterfat from 9 factories over 4 years; also minimum and maximum results obtained for each of the properties of butterfat in each month for any of the 9 factories over 4 years*

	Iodine value			Reichert value		
Month	Weighted average	Minimum	Maximum	Weighted average	Minimum	Maximum
July	39·7	34·7	41·7	28·2	26·4	31·3
Aug.	39·1	34·9	41·0	31·3	27·8	32·7
Sept.	39·1	36·0	41·7	32·2	29·1	33·9
Oct.	37·9	35·2	41·8	32·3	30·9	33·3
Nov.	34·0	32·4	37·2	31·6	30·3	32·6
Dec.	33·8	31·3	36·6	31·4	30·3	32·5
Jan.	34·3	32·3	37·6	30·7	29·3	31·8
Feb.	35·7	33·7	39·0	30·0	26·8	31·0
Mar.	36·3	33·5	40·1	28·5	27·0	30·1
April	38·3	34·8	41·1	27·0	24·4	28·7
May	39·8	37·0	42·9	25·5	23·1	27·3
June	40·2	36·4	42·7	25·7	23·7	27·1

	Saponification value			Softening point		
Month	Weighted average	Minimum	Maximum	Weighted average	Minimum	Maximum
July	224·8	222·5	228·6	33·0	31·8	34·1
Aug.	227·4	225·7	229·8	32·5	30·9	34·0
Sept.	228·8	226·9	231·5	32·4	31·2	33·5
Oct.	231·3	227·6	233·1	32·2	30·6	33·4
Nov.	232·7	230·0	233·9	32·7	31·2	33·5
Dec.	232·0	229·7	234·4	33·4	31·7	34·1
Jan.	230·6	228·0	232·8	33·7	32·6	34·5
Feb.	229·1	226·5	230·7	33·3	31·7	34·5
Mar.	227·7	225·8	229·7	33·6	32·7	34·4
April	225·3	222·4	228·3	33·6	31·4	34·4
May	222·7	220·7	225·4	33·5	31·8	34·6
June	222·5	219·8	225·6	33·4	32·3	34·2

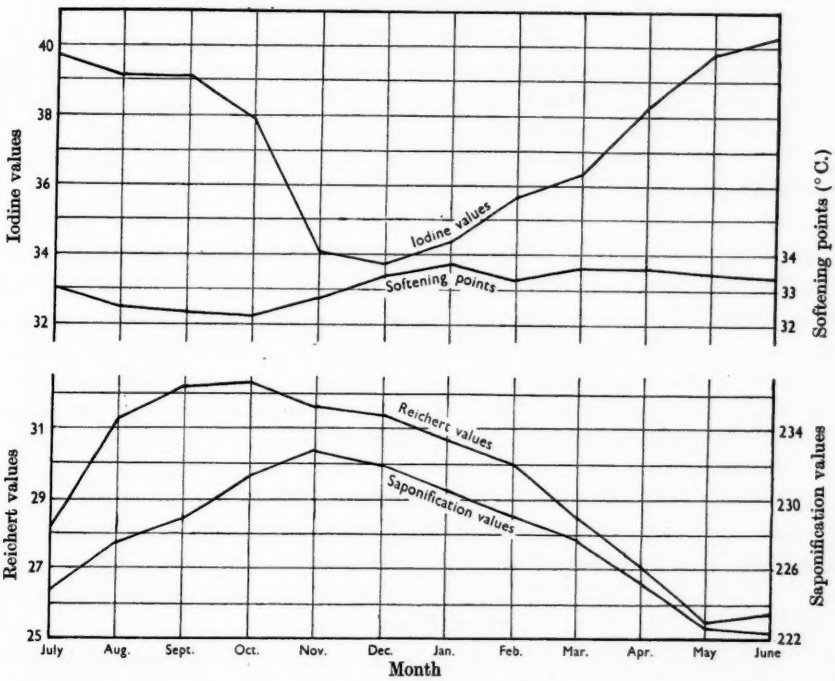

Fig. 5. Weighted monthly average iodine values, Reichert values, saponification values and softening points of butterfat from 9 factories over 4 years.

Iodine values

The average iodine values fell slowly from 39·7 in July to 37·9 in October, and then sharply to 34·0 in November. The minimum average value was reached in December, and thereafter there was a gradual rise to 40·2 in June. The weighted average iodine value for the butterfat from all districts for the 4 years was 36·7.

Reichert values

In July the average Reichert value was 28·2. There was a marked rise in August to 31·3 and a slower rise to the maximum of 32·3 in October, and thereafter a fairly steady fall to the minimum of 25·5 in May. The weighted average Reichert value for the butterfat from all districts for the 4 years was 30·4.

Saponification values

The average saponification values rose steadily from 224·8 in July to the maximum of 232·7 in November and then fell steadily to the minimum of 222·5 in June. The weighted average saponification value for the butterfat from all districts for the 4 years was 229·5.

Softening points

The average softening point fell from the value 33·0 in July to 32·2 in October, rose to 33·7 in January, and oscillated below that figure for the remainder of the year until June. The weighted average softening point for the butterfat from all districts for the 4 years was 33·1.

COMPARISON OF THE BUTTERFAT FROM THE NORTH ISLAND AND SOUTH ISLAND DISTRICTS

It has already been pointed out that the properties of the butterfats from the South Island districts showed a smaller range of variation than those of butterfats from the North Island districts. The average monthly results (not the weighted averages) for these properties for the 7 North Island factories and for the 2 South Island factories have been plotted in Fig. 6.

Iodine values

The line for the South Island butterfats shows a marked divergence from the line for the North Island butterfats during the months May to September. The reason for this has not been clearly defined but it is probably due to the greater reliance on roots, particularly turnips, as winter feed for cows in the South Island, whereas under the warmer North Island conditions more pasture is available and supplementary feeds consist in the main of hay, silage and chou moellier. It is well known that feeding of cows on turnips causes a depression in the iodine value of the butterfat.

Reichert values

The marked divergence of the South Island values from the North Island values occurred somewhat later than for the iodine values, viz. August to October.

Saponification values

The South Island values diverged from the North Island values from May to August.

Softening points

The South Island values were lower than the North Island values throughout the whole year, even during the winter period when the iodine values were lower for the South Island butterfats.

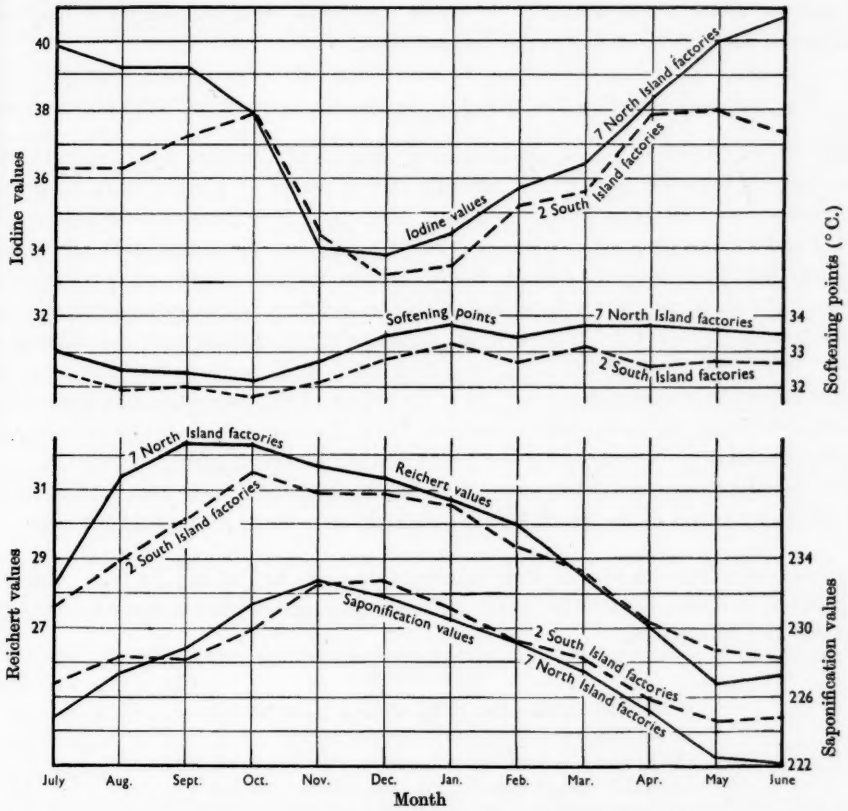

Fig. 6. Average monthly iodine values, Reichert values, saponification values and softening points (not weighted averages) of butterfat from 7 North Island factories and 2 South Island factories over 4 years.

Interrelation of the four properties of butterfat

Butterfat is a complex mixture of glycerides, and the properties measured and cited in the present paper represent averages of the properties of the individual glycerides present. The lines in Fig. 5 show that changes occur in the nature of the glycerides during the season, and that any one property, such as iodine value, is only a very rough index of the nature of the butterfat. Thus the sharp fall in iodine value in November is not accompanied by any corresponding sharp rise in softening point, and the rise in iodine value from January to June is not accompanied by a corresponding fall in softening point. The sharp increase in volatile fatty acid content from July to September is not accompanied by the expected corresponding increase in saponification value and the saponification value continues to rise for 2 months after the Reichert value has reached its peak and has

begun to fall. The lower softening point of the South Island butterfats is not in accord with the expectations from the lower iodine value of these butterfats. An explanation of the interrelationship of the figures must await a study of the detailed fatty acid composition of the butterfats at the different periods of the season. In the meantime it is clearly not possible to predict with any certainty the exact physical properties of a butterfat and the effect of the butterfat on butter properties, from the results of empirical analyses of the type described in the present paper.

Summary

Iodine values, Reichert values, saponification values and softening points of butterfats from butters collected at monthly intervals over a period of 4 years from 9 factories representative of the main butter-producing districts in New Zealand were determined. The trend of variation of any one property throughout the season was remarkably uniform, both for different factories in the one season, and for any one property in the four seasons. Weighted monthly average iodine values, Reichert values, saponification values and softening points for the butterfat from all factories over 4 years were 36·7 (33·8–40·2), 30·4 (25·5–32·3), 229·5 (225·5–232·7) and 33·1 (32·2–33·7) respectively. The minimum iodine value occurred in midsummer, i.e. at the season of the year when maximum values are reported for northern hemisphere butters. The iodine values for South Island butterfats diverged markedly from those for the North Island butterfats during the winter, i.e. at the time when turnips are fed to cows in the South. In spite of these lower iodine values, the softening points of the South Island butterfats were lower throughout the year.

The authors desire to express their thanks to Prof. W. Riddet and Dr W. J. Wiley for suggestions in connexion with the work, and to the Managers of the 9 factories for arranging the dispatch of samples.

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372. CONTROL OF THE MOISTURE CONTENT AND 'BODY-FIRMNESS' OF CHEDDAR CHEESE

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Finest-quality New Zealand Cheddar cheese when officially examined 14–21 days after manufacture must satisfy the requirements of expert judges in respect to several specific qualities, viz. flavour, body, texture (closeness), colour and finish. A desirable body implies the correct firmness in consistency when a piece of the cheese is kneaded in the fingers and a smooth waxy 'feel' which persists as the kneading progresses. These two qualities—firmness of the correct degree, and smoothness—do not necessarily go together. A cheese may have the desired firmness and yet lack smoothness and vice versa. Of the two qualities, smoothness is the harder to attain regularly and with certainty because the factors which control it are not clearly understood. The rate and extent of acid development during the cheesemaking process have something to do with it, but there are other factors as yet undefined. In certain circumstances smoothness is difficult to attain owing apparently to some abnormality in the qualitative nature of the cheese milk. Firmness of body on the other hand is determined mainly by the relative amounts of moisture, fat, and protein in the cheese and by the extent to which the protein has been altered by enzyme action (variations in hardness of fat are hardly significant in their influence on cheese firmness). These factors which determine the firmness of cheese may for convenience be expressed in another way, viz.:

(*a*) Water content of the non-fatty cheese solids, i.e. the proportion of water to protein (W.N-F.S.).

(*b*) Fat content of the water-free cheese solids, i.e. the proportion of fat to protein (F.W-F.S.).

(*c*) pH of the cheese, which influences enzyme action.

(*d*) Content of salt in the cheese moisture, which influences the physical state of the cheese protein.

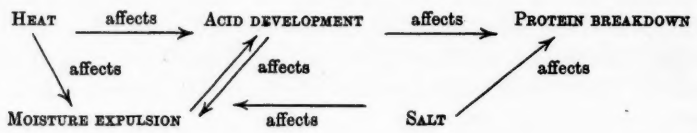
The temperature of the cheese at the time of examination may be included as a fifth factor but a constant temperature may be assumed for our present purpose.

The application of rheological methods[1, 2] to the determination of the physical characters of cheese has shown that there is a strong correlation between moisture content and firmness of body, and this finding confirms all experience with subjective grading of cheese. Provided that the milk protein is normal, the acidity development in the making process is regular, leading to an optimal final pH in the curd, and that the salt content of the cheese curd is within certain narrow limits, then it is almost self-evident that the proportions of moisture, fat and protein must determine the firmness of the cheese. Further, since the proportion of protein to fat is settled by the composition of the milk as drawn from the cow (in the absence of addition or removal of cream), it follows that moisture in the cheese is the major factor which tends to vary in accordance with the nature and composition of the cheese curd and with the treatment of the curd in the cheese vat. Adjustment of moisture content by appropriate treatment during the manufacturing process is thus the cheesemaker's only method of controlling firmness in the

final cheese. This paper deals with the effect of milk composition on the moisture-retaining properties of cheese curd, and with the methods found necessary under various circumstances to arrive at the optimum moisture content in the non-fatty cheese solids (W.N-F.S.).

Moisture control in the cheesemaking process

Cheese manufacture is essentially the removal of moisture from cheese curd under certain defined conditions of acid development. The main difficulties encountered by the cheesemaker are those concerned with defining the correct acidity conditions at different stages of the process and with achieving them constantly from day to day. These difficulties are enhanced during periods when the cheese milk alters from day to day in composition (quantitative or qualitative). A balance between acid development and curd moisture must be maintained throughout the process and the interdependence of the various factors make control very complicated. Moisture is expelled by contraction of the curd under the influence of both heat and increase in acidity; acid development is influenced by moisture content and temperature; and moisture expulsion in the last stages in the cheese vat is influenced by salt which also affects subsequent acid development. Finally, the acid and salt content of the raw cheese curd affect its enzymic breakdown and hence its ultimate physical properties:



Regularity from day to day

Experience has shown that if the cheese is to have the desired physical qualities it is supremely important that moisture and acid should be in the correct balance at the moment when the bulk of the whey is drained from the curd (drying time or piling time). In view of this Scott Blair and his associates (3, 4) have endeavoured to measure and define the physical properties which should be possessed by the curd at this crucial time. They thus hope to provide a criterion for the control of the making process even though the rate of acid development or the character of the cheese curd may vary from day to day. In New Zealand the approach to cheesemaking control has been made from a different angle. Since it was evident that acid development in the cheese vat was the factor most liable to vary from day to day in a capricious manner we attempted to gain a greater control of starter cultures and thus to obtain a relatively steady rate of acid development. When this was to a great extent accomplished (5) it became possible, *provided no alteration in the nature or composition of the milk occurred*, to discover by trial and error the best manufacturing procedure. The drawback to the method is that at least a fortnight elapses before the result of a given system can be observed in the cheese. Moreover, whenever a significant alteration in milk occurs, adjustment of the manufacturing procedure must be made again by trial and error. A knowledge of the art of cheesemaking makes possible an intelligent estimate of the changes necessary under any given circumstances, but it would be an improvement if a system such as that visualized by Scott Blair could be

added to the advantages of a constant rate of acid production from day to day. In the meantime with starters of regular vitality it is at least possible to manufacture cheese according to a routine and to get reproducible results for quite long periods. This was the main factor which made possible the work to be described. Before single-strain starters of constant activity were available for use in our experimental factory, variability in acid production from day to day made the accumulation of a sufficient number of comparable results extremely difficult. Another requirement in cheese experiments is of course a milk supply of good bacteriological quality; otherwise irregularities in acid development occur as a result of the presence of acid-producing contaminants. Pasteurization of the cheese milk by destroying contaminant bacteria also gives greater regularity in acid production as has been realized in New Zealand for many years and more recently (6) in America. The cheese milk used in all the experiments to be described was of high quality (reductase test always over 5 hr. and usually over 7 hr.) and it was flash-pasteurized at 155° F. Thus the constant activity of the starters used, the high quality of the milk and its pasteurization all contributed to the basic conditions necessary for replication of cheesemaking experiments over a period of weeks. Under these conditions the aim of the experiments was to define more precisely the effects of differences in milk composition on the moisture-retaining properties of cheese curd and to find (by trial and error) the changes necessary with different milks to produce cheese with the same content of moisture in the non-fatty solids.

EXAMINATION OF EXPERIMENTAL CHEESES

All the cheeses were examined and analysed 14 days after manufacture, this being the standard time under commercial conditions in New Zealand. Judgements of firmness of body were made subjectively by a panel of observers who were kept in ignorance of the identity of the various cheeses. In the absence of rheological measurements, however, the subjective judgements of firmness were recorded merely for comparison with the cheese-moisture figures. They accorded with the general experience that moisture in the non-fatty portion of the cheese (W.N-F.S.) determines the firmness provided that pH is normal and fat content falls within certain limits. It is more satisfactory, however, to base the argument on the figures for W.N-F.S. in the experimental cheeses because statistical analysis is possible. Even the chemical analysis of cheese is subject to relatively large possible errors. Fat in the cheeses was determined by the Babcock method; more accurate analysis could not be carried out owing to limitation of staff. The possible error in the Babcock fat-estimation is $\pm 0{\cdot}6$ which gives a possible error in the calculated W.N-F.S. of $\pm 0{\cdot}4$. The possible error in moisture estimation due to errors of sampling and analysis is $\pm 0{\cdot}5$ which gives a possible error in the calculated W.N-F.S. of $\pm 0{\cdot}75$. The total possible error in W.N-F.S. is therefore $\pm 1{\cdot}15$ (7). Although the probable error (which is unknown) must be smaller than this maximum possible error it may still be large in relation to the differences observed between pairs of experimental cheeses. Hence it was necessary to include relatively large numbers of pairs of cheeses in each experiment and to determine statistically the significance of the difference between the mean W.N-F.S. figures. Wherever a difference was not very evidently significant by inspection, Student's 't' was calculated and the level of significance determined. The values of 't' so obtained are given in the tables.

The effect of variations in fat and casein content of cheese milk on the moisture-retaining properties of cheese curd

(1) *Variations in fat content*

It has been recognized for many years by cheesemakers that milks of different fat content demand differences in treatment in the cheese vat if cheese of normal body firmness is always to be produced. Sammis (8) clearly showed that 'moisture is removed more rapidly from curds with the smaller content of fat. The fat by its presence somewhat delays the separation of moisture from casein'. Van Slyke & Price (9) state that milk with a low percentage of fat forms curd which needs to be cooked at a lower temperature (i.e. it loses moisture more readily) than curd from milk with a high percentage of fat. There has been, however, a good deal of misapprehension about this matter of high- and low-fat milk. The findings quoted above are strictly true only when the milks compared are the same in composition except for the proportion of fat. As will appear later there are circumstances under which a high-test milk from Jersey cows forms a curd which has a lower moisture-retaining tendency than a low-test milk from Friesian cows. The influence of fat content upon moisture retention can be investigated only where the milks compared are otherwise of similar composition or where the difference in fat content is due to removal or addition of fat (commercially called standardization). The experiments of Sammis showed in effect that some moisture is bound by the fat in milk and is less readily expelled from cheese curd than the rest of the moisture. McDowall (10) relating the casein/fat ratio of milk to yield of cheese and using the results obtained in practice from more than a thousand commercial cheese vats found that cheese yield could be calculated from casein and fat content of the milk by applying a formula in which the factor ($1{\cdot}19 \times$ fat) was included. This implies that fat contributes 1·19 times its own weight to the cheese or in other words that fat carries moisture into the cheese.

Addition of fat to cheese milk

Whitehead (11) found that the addition of extra fat to cheese milk (in the form of whey cream) gave an extra yield of cheese equal to approximately 1·2 times the weight of fat added. This corresponds well with McDowall's formula. In these experiments no variation in cheesemaking procedure was made between the experimental and control vats. The cheese containing added fat showed a higher W.F-N.S. and a softer body than the control cheese. The softer body was due partly to extra moisture and partly to extra fat. The results were clear-cut without statistical analysis. Out of twenty-six occasions when a control vat was compared with an experimental vat to which whey fat had been added in such an amount as to give an average of 2·06 increase in the F.W-F.S. of the cheese, there were only three occasions when the W.N-F.S. of the experimental cheese was lower than that of the control cheese. On the average the W.N-F.S. of the experimental cheese was 1·06 higher (control cheese 53·87 W.N-F.S.).

Abstraction of fat from cheese milk

Experience throughout the world on the handling of cheese milk from which fat has been removed has confirmed Sammis's finding in 1910 that, treated similarly to the corresponding whole milk, such 'standardized' milk forms a curd which loses moisture more readily than whole milk curd. Thus if no adjustment is made in the cheesemaking process the standardized cheese is lower in W.N-F.S. and firmer in body. Many experiments

have been carried out at this Institute over the course of the last 15 years with the object of determining the appropriate vat procedure necessary to produce a cheese of normal W.N-F.S. and firmness from standardized milk. The variations in technique which are necessary in order to reduce moisture loss from a low-fat curd are

(*a*) A reduced cooking (scalding) temperature.

(*b*) A smaller amount of 'dry-stirring' of the curd when the whey is removed.

(*c*) A smaller proportion of salt.

The question of the order of importance of these variations is not finally settled. Table 1 gives typical manufacturing details at different periods of the dairying season (spring and autumn) for comparable whole and standardized milks, where the processes had finally been adjusted so as to give cheeses of almost identical W.N-F.S.and similar firmness of body. The significant differences in treatment are in heavy type.

Table 1. *Manufacturing procedures with whole milk and 'standardized' milk necessary to give same* W.N-F.S. *in the cheese*

	7 November 1944		7 March 1945	
	Whole	Standardized	Whole	Standardized
Fat (%)	4·5	3·8	4·8	3·9
Casein (%)	2·75	2·75	2·80	2·80
Ratio (casein/fat)	0·61	0·72	0·58	0·72
Starter (%)	1·25	1·25	1·125	1·0
Ripening (min.)	30	30	30	30
Coagulation (min.)	30	30	30	30
Acid cut	0·095	0·095	0·09	0·085
Cooking temp. (° F.)	**100**	**99**	**100**	**97**
Acid dry	0·185	0·19	0·175	0·17
Dry stirs	**2**	**1**	**1**	**0**
Time in whey (hr. min.)	2 45	2 50	2 40	2 40
Acid salt	0·84	0·83	0·82	0·85
Time dry to salt (hr. min.)	2 35	2 45	2 20	2 20
Salt (%)	**2·5**	**2·25**	**2·5**	**2·125**
Cheese 14 days:				
W.N-F.S.	53·1	53·0	54·9	54·1
F.W-F.S.	54·5	51·1	55·5	50·0
pH	4·87	4·91	4·84	4·87

Table 2 gives the composition (at 14 days of age) of sixteen comparable pairs of cheeses made from high fat and standardized milks in the 1946–7 season. Eighty gallons of milk were used in the experimental vats. The fat content of the standardized cheeses was reduced to 52 F.W-F.S. The results show that the cheesemaker was successful in attaining the same W.N-F.S. in each pair of cheeses by adopting the methods described above to retain moisture in the standardized curd. The comparable pairs of cheeses when examined 14 days after manufacture were of the same firmness of body, and there was no significant difference in the general quality of the pairs when they were 4 months old.

It is thus quite clear that milk fat tends to retain moisture in cheese curd. Where two curds in cheese vats differ in fat content and other factors are the same (i.e. casein content and rate of acid production) the curd with the lower fat content demands treatment designed to retain moisture as compared with the curd of higher fat content.

(2) *Variations in casein content*

Adjustment of the casein content of a cheese milk is for obvious reasons not easily possible. And where variations in casein content between various milk supplies occur it is usually accompanied by a variation in fat content. It is therefore not so simple to

decide what influence, if any, casein content has upon the moisture-holding properties of cheese curd as it is to determine the influence of fat. A certain amount of information can, however, be deduced from cheesemaking experiments with milk from different breeds of

Table 2. *Composition of whole-milk and standardized milk cheese. Manufacture designed to give same* W.N-F.S.

	F.W-F.S.			W.N-F.S.		
Date	Cont.	Stand.	Diff.	Cont.	Stand.	Diff.
30 Sept. 46	59·5	53·5	4·0	55·6	54·3	1·3
1 Oct. 46	58·4	52·1	6·3	53·5	53·7	−0·2
2 Oct. 46	56·5	52·8	3·7	53·9	54·0	−0·1
4 Oct. 46	58·4	53·7	4·7	54·2	54·6	−0·4
4 Nov. 46	55·6	52·2	3·4	53·1	54·0	−0·9
5 Nov. 46	56·1	51·9	4·2	53·0	53·4	−0·4
6 Nov. 46	57·7	54·3	3·4	53·3	54·7	−1·4
7 Nov. 46	56·9	52·6	4·3	54·5	54·7	−0·2
8 Nov. 46	56·7	52·6	4·1	54·1	55·6	−1·5
9 Dec. 46	56·1	52·2	3·9	53·0	52·8	0·2
10 Dec. 46	56·2	52·0	4·2	53·2	53·5	−0·3
11 Dec. 46	55·8	51·2	4·6	53·5	53·1	0·4
12 Dec. 46	56·2	51·6	4·6	54·2	53·8	0·4
13 Dec. 46	56·8	52·4	4·4	53·4	52·1	1·3
16 Dec. 46	54·6	50·5	4·1	54·3	53·8	0·5
17 Dec. 46	56·4	52·3	4·1	52·6	53·1	−0·5
18 Dec. 46	55·4	51·2	4·2	52·7	53·0	−0·3
19 Dec. 46	54·8	52·4	2·4	52·5	53·2	−0·7
20 Dec. 46	56·5	53·1	3·4	54·8	54·5	0·3
Average	56·6	52·3	4·3	53·7	53·8	0·1

Significance of difference between means of W.N-F.S., $t = 0{\cdot}521$. For 18 degrees freedom, $t = 2{\cdot}1$ at 5% level. Therefore difference not significant.

Table 3. *Composition of cheese from low-test and high-test milks. Manufacturing procedure the same for both*

	F.W-F.S.			W.N-F.S.		
Date	Low	High	Diff.	Low	High	Diff.
5 Oct. 42	52·9	54·9	2·0	53·2	52·6	0·6
6 Oct. 42	51·4	57·0	5·6	54·0	52·9	1·1
7 Oct. 42	50·9	55·8	4·9	53·4	52·3	1·1
8 Oct. 42	52·0	55·9	3·9	53·2	52·5	0·7
9 Oct. 42	50·9	55·7	4·8	53·0	52·4	0·6
12 Oct. 42	53·5	55·8	2·3	54·3	53·1	1·2
14 Oct. 42	50·4	54·6	4·2	53·7	51·6	2·1
15 Oct. 42	49·5	52·6	3·1	52·9	51·1	1·8
16 Oct. 42	50·0	56·9	6·9	52·1	51·5	0·6
19 Oct. 42	50·6	54·8	4·2	53·2	51·1	2·1
21 Oct. 42	50·4	55·9	5·5	53·7	53·2	0·5
22 Oct. 42	52·9	55·3	2·4	53·3	53·0	0·3
23 Oct. 42	51·8	54·7	2·9	53·7	53·4	0·3
27 Oct. 42	53·3	55·9	2·6	53·6	52·5	1·1
28 Oct. 42	50·0	57·7	7·7	53·0	52·7	0·3
29 Oct. 42	52·4	55·2	2·8	54·0	52·7	1·3
30 Oct. 42	52·1	55·5	3·4	53·1	51·7	1·4
Average	51·5	55·5	4·0	53·4	52·4	1·0

Significance of difference between means of W.N-F.S., $t = 7{\cdot}0$. For 16 degrees freedom, $t = 2{\cdot}9$ at 1% level. Therefore difference highly significant.

cattle. Experiments conducted at this Institute over the course of years have indicated that curd from Jersey milk (high-fat and high-casein) loses moisture in the cheesemaking process more readily than curd from Friesian milk (low-fat and low-casein).

Table 3 gives the composition of seventeen pairs of cheeses (80 gal. vats) made from Jersey and Friesian milks in the 1942–3 season. In this experiment no attempt was made

to make allowance for the different moisture-retaining characteristics of the two curds. The only difference in the treatment of the two milks was that necessary to produce a final curd of the same pH in the same time. Jersey milk has a higher natural acidity and it was found necessary to dry the curd at a correspondingly higher figure. Also acidity development in Jersey milk tends to be slightly slower than in Friesian milk, so a slightly larger proportion of starter was sometimes used. Otherwise the two vats were treated similarly; they were cooked to the same temperature, the curds were stirred to the same extent and the same proportion of salt was used. The statistically lower average W.N-F.S. figure for the Jersey cheeses shows quite clearly that the Jersey curd lost moisture more readily than the Friesian curd. The Jersey cheeses were correspondingly firmer in body.

Table 4. *Composition of cheese from low-test and high-test milks. Manufacture designed to give same* W.N-F.S.

	F.W-F.S.			W.N-F.S.		
Date	Low	High	Diff.	Low	High	Diff.
14 Dec. 42	52·3	56·1	3·8	53·8	53·6	0·2
15 Dec. 42	50·2	55·4	5·2	53·2	53·2	0·0
16 Dec. 42	53·9	55·1	1·2	53·5	52·5	1·0
17 Dec. 42	52·7	54·9	2·2	53·8	52·2	1·6
18 Dec. 42	52·9	55·3	2·4	53·3	53·2	0·1
21 Dec. 42	54·7	55·2	0·5	54·6	52·8	1·8
22 Dec. 42	51·6	55·6	4·0	53·3	52·2	1·1
23 Dec. 42	51·5	56·5	5·0	52·7	52·8	−0·1
5 Jan. 43	51·9	55·4	3·5	53·0	52·3	0·7
6 Jan. 43	51·9	55·7	3·8	53·0	52·8	0·2
7 Jan. 43	53·8	55·5	1·7	54·1	53·3	0·8
8 Jan. 43	51·6	55·4	3·8	52·9	53·1	−0·2
10 Mar. 43	50·2	56·7	6·5	53·4	53·5	−0·1
11 Mar. 43	50·9	56·1	5·2	53·7	54·5	−0·8
12 Mar. 43	52·2	55·7	3·5	54·1	53·7	0·4
15 Mar. 43	49·6	54·9	5·3	53·9	53·0	0·9
16 Mar. 43	54·2	56·2	2·0	55·3	53·5	1·8
17 Mar. 43	55·7	56·5	0·8	54·6	54·0	0·6
18 Mar. 43	52·5	55·7	3·2	54·2	53·6	0·6
19 Mar. 43	52·3	55·3	3·0	54·3	53·8	0·5
22 Mar. 43	51·4	56·8	5·4	53·9	54·2	−0·3
23 Mar. 43	52·2	55·8	3·6	53·7	54·7	−1·0
24 Mar. 43	53·5	56·3	2·8	54·8	54·3	0·5
25 Mar. 43	49·0	54·1	5·1	54·1	54·0	0·1
26 Mar. 43	51·9	54·4	2·5	53·5	54·8	−1·3
Average	52·2	55·6	3·4	53·8	53·4	0·4

Significance of difference between means of W.N-F.S., $t=2·5$. For 24 degrees freedom, $t=2·79$ at 1% level; 2·06 at 5% level. Therefore difference significant but not highly significant.

In order to compensate for this differential loss of moisture from Jersey and Friesian curds it has been found necessary to vary the cheese-manufacturing procedure in a manner similar to that necessary when milks of different fat content are being handled except that in this instance the measures designed to retain moisture (lower cooking temperature, less dry-stirring and lesser proportion of salt) have to be applied to the Jersey milk in spite of the fact that it has a higher fat content than the Friesian milk. Table 4 gives the details of composition of twenty-five comparable pairs of cheeses made from Jersey and Friesian milk respectively. In these experiments an attempt was made by adjustment of cooking temperature and dry-stirring procedure (but not by alteration of salt proportion) to produce cheeses of similar W.N-F.S. Typical manufacturing details at different periods of the dairying season are given in Table 5, the significant differences being in heavy type. Consideration of the results shows that even when differences up to 4° F. in cooking

temperature were applied, coupled with a significant difference in stirring treatment, the cheeses made from Friesian milk still tended to have a higher W.N-F.S. The difference between the mean W.N-F.S. figures was still significant at the 5% level and in all probability a larger proportion of salt should have been added to the Friesian curd in order to reach substantial equality in W.N-F.S. in the pairs of cheeses.

The above results make it quite clear that low-fat Friesian curd retains moisture during the cheesemaking process more tenaciously than high-fat Jersey curd. Since fat has a moisture-retaining tendency it follows that the protein fraction of the Friesian curd must have an extra moisture-retaining tendency which more than compensates for the lesser amount of fat present. This moisture-retaining tendency of Friesian curd may, however, be more apparent than real. The phenomenon may actually be merely a reflexion of the fact that Friesian curd contains initially a higher proportion of water and lower proportion of casein. In the cheesemaking process it is thus necessary (in order to arrive at a given W.N-F.S. in the cheese) to remove a greater proportion of moisture from a

Table 5. *Manufacturing procedure with Friesian and Jersey milks necessary to give same* W.N-F.S. *in the cheese*

	14 December 1942		24 March 1943	
	Friesian	Jersey	Friesian	Jersey
Fat (%)	3·5	4·95	4·1	5·6
Casein (%)	2·40	2·85	2·60	3·15
Ratio (casein/fat)	0·68	0·57	0·63	0·56
Starter (%)	1·0	1·0	0·875	0·875
Ripening (min.)	30	30	30	30
Coagulation (min.)	45	35	35	30
Acid cut	0·08	0·095	0·08	0·095
Cooking temp. (° F.)	100	97	102	98
Acid dry	0·16	0·18	0·16	0·18
pH dry	5·83	5·77	5·75	5·79
Dry stirs	2	2	3	1
Time in whey (hr. min.)	2 50	2 52	2 55	3 0
Acid salt	0·80	0·80	0·81	0·81
Time dry to salt (hr. min.)	2 15	2 3	2 40	2 25
Salt (%)	2·5	2·5	2·5	2·5
Cheese 14 days:				
W.N-F.S.	53·8	53·6	54·8	54·3
F.W-F.S.	52·3	56·1	53·5	56·3
pH	4·86	4·87	4·90	4·89

Friesian curd than from a Jersey curd in the same time (assuming that the whole time-schedule of the process is not to be altered). On the average about 25% more whey has to be expelled from the Friesian curd.

The difficulty in inducing an adequate speed of whey expulsion from a Friesian curd may therefore be due either to some difference in the qualitative nature of the rennet coagulum (in other words in the nature of the casein) of Friesian curd as compared with the Jersey curd or merely to the extra amount of moisture which has to be removed from Friesian curd within a given time. Sammis (8) carried out cheesemaking experiments on milk to which various proportions of water had been added and came to the conclusion that curd containing additional water (or conversely a smaller proportion of casein) lost the extra moisture rapidly in the cheese vat and finally reached the same W.N-F.S. in the cheese without the necessity for the application of any different treatment. This would tend to indicate that the differences found in our experiments as between Jersey and Friesian milks were due to some qualitative difference between the proteins of the two milks which affected moisture-retaining tendency.

Adjustment of casein content

An attempt was made to obtain further evidence on this point by adjusting Jersey milk by water addition and fat abstraction to the same fat and casein content as Friesian milk and then comparing the cheesemaking properties of the two milks. Table 6 gives the compositional details of fourteen comparable pairs of cheeses (80 gal. vats) made from Friesian milk and from Jersey milk adjusted to the same gross composition and treated similarly in the cheese vat. The results indicate that there was no significant difference in moisture retention by the two curds. On a few occasions (shown in the table) the Jersey milk adjusted in fat content but not in casein content was made into cheese by exactly the same procedure in a third vat. It is quite obvious without statistical treatment that the W.N-F.S. of these cheeses was significantly lower than that of the other two series of cheese. They were also markedly firmer to the feel. These results are not conclusive. They are too few in number and may be unreliable by reason of the fact that the proportions

Table 6. *Composition of cheese from low-test milk, high-test milk adjusted to same fat content and high-test milk adjusted to same casein and fat content. Manufacturing procedure same for all*

	F.W-F.S.				W.N-F.S.			
Date	Low test	High test adjusted F. and C.	Diff.	High test adjusted F.	Low test	High test adjusted F. and C.	Diff.	High test adjusted F.
24 Sept. 46	52·3	52·9	0·6	—	51·9	53·1	1·2	—
25 Sept. 46	52·7	52·3	−0·4	—	54·8	52·0	−2·8	—
7 Oct. 46	51·3	52·2	0·9	49·3	54·3	54·8	0·5	53·8
8 Oct. 46	52·3	53·8	1·5	49·5	54·0	54·7	0·7	51·2
25 Nov. 46	53·5	53·3	−0·2	51·3	55·2	54·9	−0·3	53·2
26 Nov. 46	52·9	53·1	0·2	50·0	53·6	54·4	0·8	51·9
27 Nov. 46	50·7	50·2	−0·5	46·6	54·2	53·3	−0·9	52·1
28 Nov. 46	52·6	53·2	0·6	48·6	53·5	52·6	−0·9	51·2
29 Nov. 46	49·7	51·6	1·9	45·6	53·5	53·9	0·4	51·2
3 Feb. 47	54·6	53·9	−0·7	—	54·2	55·0	0·8	—
4 Feb. 47	54·2	54·4	0·2	—	54·4	53·9	−0·5	—
5 Feb. 47	53·7	51·2	−2·5	—	54·6	54·0	−0·6	—
6 Feb. 47	52·9	53·0	0·1	—	54·2	54·3	0·1	—
7 Feb. 47	53·5	53·8	0·3	—	55·2	54·8	−0·4	—
Average	52·6	52·8	0·2	48·7	54·1	54·0	−0·1	52·1

Significance of difference between means of W.N-F.S., $t = 0{\cdot}36$. For 13 degrees freedom, $t = 2{\cdot}65$ at 5% level. Therefore difference not significant.

of the water-soluble constituents of the milk were altered by the addition of water. The watered-milk cheeses were slightly higher in pH at 14 days than the comparable Friesian cheeses probably as a result of a lower lactose content when the curd was salted. They were consequently slightly firmer in body than would have been predicted from the W.N-F.S. figures but the variation from normal was remarkably slight. The results indicate that to some extent at least the difference between Jersey and Friesian curds may be one of water content. This is opposed to the findings of Sammis. Further work is projected on the effect of the addition of lactose solution and sweet whey to Jersey milk in an attempt to obtain more reliable evidence on the point at issue.

DISCUSSION

A consideration of all the results detailed above leads to a clearer picture of the effect of variations of fat and casein content of milk on the behaviour of curd in the cheesemaking process. Fat has a moisture-retaining effect, hence the higher the fat content of a given

milk the more difficulty there is in reducing the moisture in the curd and cheese to a given value (the average W.N-F.S. at 14 days of age of 70 cheeses considered normal in firmness in the present experiments was 54% (range 55·7–52·1)). Casein content also has an effect on the ease of expulsion of moisture from curd. Under normal circumstances significant variations in casein content are usually encountered as a result of the segregation of milk from cows of different breeds (e.g. Jersey and Friesian) and the difference in casein content is accompanied by a difference in fat content. Friesian milk of low casein content forms a curd from which moisture is more difficult to remove (down to a given value) than it is from Jersey curd in spite of the fact that the latter has more moisture-retaining fat present in it. Evidently this is due either to the fact that low-casein curd needs to lose more moisture within a given time or to some difference in its qualitative nature as compared with high-casein curd. At least some of the effect is due to the first alternative but more evidence is needed before the question can be definitely settled.

So far as the cheesemaker is concerned the position may be summarized as follows. In order to produce cheese of a given moisture content in the non-fatty solids:

(i) 'Low-fat curd' must be 'cooked' at a higher temperature, stirred more, and salted more heavily than 'high-fat curd' if the low-fat content is a natural phenomenon associated with the breed of cow from which the milk is produced and with a lower casein content. The lower casein content is the really significant feature of such milk and dictates the treatment required.

(ii) 'Low-fat curd' must be 'cooked' at a lower temperature, stirred less and salted more lightly than 'high-fat curd' if the casein contents of the two curds are similar.

It is of course possible to have two milks of such compositions that low-fat content is associated with low-casein content and high-fat content with high-casein content in such a way that the two curds formed have almost equal moisture-retaining tendency, the 'fat effect' in one milk balancing the 'casein effect' in the other. This condition with a very low-fat Friesian milk and a very high-fat Jersey milk is sometimes approached although more usually the 'casein effect' in Friesian milk overshadows the 'fat effect' in the Jersey milk.

Summary

Cheesemaking experiments were carried out to investigate the moisture-retaining characteristics of curd made from milks of varying fat and casein content. The results indicate:

(*a*) That milk fat helps to retain moisture in a cheese curd. The higher the proportion of fat present the more drastic the treatment required in the cheese vat to reduce the moisture content of the finished cheese to the desired level. A higher cooking temperature, more dry-stirring of the curd and a larger proportion of salt are all necessary with rise in fat content of the curd.

(*b*) That curd formed from milk of a low-casein content (from Friesian cows) retains moisture in the cheesemaking process more tenaciously than curd from milk of a high-casein content (from Jersey cows).

The two effects given under (*a*) and (*b*) tend to neutralize each other but the 'casein effect' is usually more powerful than the 'fat effect'. It is not possible to decide from the evidence available at present whether the 'casein effect' is quantitative only, more moisture having to be removed from a low-casein curd, or whether there is also a qualitative difference between the caseins in Jersey and Friesian milk with an associated difference

in moisture-retaining capacity. The results of a few experiments indicated that the quantitative aspect plays a large part but more evidence is needed to clarify this point.

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373. INHIBITORY STRAINS OF LACTIC STREPTOCOCCI AND THEIR SIGNIFICANCE IN THE SELECTION OF CULTURES FOR STARTER

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I. Introduction

Rogers (1) reported in 1928 that lactic streptococci could inhibit the growth of other lactic streptococci and of *Lactobacillus bulgaricus*. Whitehead & Riddet (2) were the first to isolate streptococci capable of inhibiting the growth of starter cultures during cheesemaking and to show that these streptococci caused marked delays in acid development. They investigated the spasmodic appearance of 'slow' vats during cheddar cheese manufacture and demonstrated that the streptococci responsible for the so-called 'non-acid' milk produced a substance capable of restraining the growth of lactic streptococci. This inhibitory substance was not destroyed when the 'non-acid' milk was heated to 212° F. for 30 min. Whitehead (3) investigated the nature of the inhibitory principle, produced by two different strains and suggested that it was of a protein nature. He classified one of these strains as *Str. cremoris* and the other he considered resembled *Str. mastitidis*. The acid production of different strains of *Str. cremoris* was shown to be inhibited in variable degrees on the addition of 10% 'non-acid' milk (4).

Meanwell (5) reported in 1943 the occurrence of 'non-acid' milk as a cause of 'slowness' in cheesemaking and found that the majority of 'slow' milks were of poor bacteriological quality. Oxford (6) working with two strains of inhibitory streptococci, isolated by Meanwell, observed that of the organisms he tested, the most susceptible to the inhibitory principle (*Str. cremoris*) was practically indistinguishable from the inhibitory strains themselves. He recorded further studies on the biochemical nature of the inhibitory substance and suggested the name diplococcin.

Attempts to concentrate the inhibitory substance are reported by Mattick & Hirsch (7) who obtained from *Str. lactis* a product of high potency which completely inhibited the growth of many organisms at high dilution.

II. Experimental

In the course of other investigations when attempts were made to induce bacteriophage action in strains of *Str. cremoris* and *Str. lactis* inhibitory strains were isolated from starters themselves. A method of demonstrating lysogenicity, if it occurs amongst the lactic streptococci, was sought within strains already selected as suitable for starter, so that the phages active against the different strains might be detected prior to their use. Strains were grown together in pairs in broth medium which was then Seitz filtered. The filtrate was divided and added to two tubes of broth, each of which was seeded with one of the two strains originally grown together. It was found that failure of either organism to grow in the presence of this filtrate was due to the presence of an inhibitory substance produced by the other organism of the original pair and not, as had been hoped, to phage action.

Several of the strains obtained from starters themselves as well as freshly isolated strains of lactic streptococci were found to be inhibitory. It was decided to investigate more fully the occurrence of such strains and the extent of inhibition likely to be produced, since, in the selection of strains for starter, especially where several strains are to be used in the same vat, the exclusion of inhibitory strains is important.

Source of lactic streptococci and method of determining whether any produced an inhibitory substance

The total of 276 strains of lactic streptococci examined consisted of two main groups:

(1) 220 'cultivated' strains of *Str. cremoris* originating from commercial starters including 21 substrains from starter.

(2) 56 'wild' strains isolated from naturally souring milk of poor bacteriological quality. Since they were selected for use as starter cultures, they had the property of rapidly producing acid in milk.

To detect inhibitory strains, each was grown in a tube of yeast-dextrose broth (Y.D.B.)* for 18 hr. at 30° C., and then killed by immersion in boiling water for 5 min. One ml. (i.e. 10%) of the heated culture, after rapidly cooling, was added to each of two tubes containing 10 ml. of Y.D.B. previously seeded with two other strains of lactic streptococci, selected at random. When both these strains failed to grow, the strain under test was regarded as inhibitory, but, if only one of these strains grew, the heated culture was tested against two additional strains of lactic streptococci.

METHOD OF ESTIMATING INHIBITORY POWER OF LACTIC STREPTOCOCCI

Two tests were used for comparing the strength of the inhibitory substance produced by the strains selected.

(1) *Dilution test*

Cultures of inhibitory strains were heated and, if not tested immediately, were stored at 3° C. for not more than three days. The strength of inhibitory substance produced by the different cultures was examined, using calibrated dropping pipettes, by using a series of dilutions ranging from 1/10, 1/20 to 1/1280. The test organism (24 hr. culture) grown in Y.D.B. was diluted in Ringer's solution ($\frac{1}{4}$ strength) to 1/10,000 and then further diluted in the test medium so that the concentration of the original culture was 1/100,000.

The details of preparing the dilutions of inhibitory culture were modifications of the method adopted by Hirsch (8) using only small quantities of inhibitory substance. The test was carried out in Y.D.B. or litmus milk. When the test was made in Y.D.B. preparatory dilutions of the heated cultures were made in the same medium. Similarly, when the inhibitory strains had been grown and were to be tested in litmus milk, the preparatory dilutions were made in litmus milk. In some of the preliminary examinations when the test medium was litmus milk, the inhibitory strain had been grown in Y.D.B., and the preparatory dilutions were made in Y.D.B. Additions of sterile Y.D.B. were then made to certain of the dilutions of the test series to compensate for the stimulatory effect of yeast-dextrose medium on cultures of lactic streptococci.

The series of dilutions were incubated at 30° C. and readings taken at 16 and 24 hr. Throughout the tables, the highest dilution in which no growth of the test organism was apparent after incubation for 16 or 24 hr. is recorded.

* 2% peptone, 1% lemco, 0·5% dextrose, 0·5% salt, 0·3% Yeastrel.

Although the inhibitory substance delayed growth of the test organism in higher dilution in Y.D.B. than in litmus milk, both media were retained for comparison in the dilution test (Table 1). Information regarding the inhibitory action on starter strains growing in milk was desired and the use of litmus milk as a test medium, even if less sensitive, was useful for this purpose.

Table 1. *Sensitivity of strain* 1P5 *compared with* 144F *for use as test organism and of broth with milk as test medium*

Test medium	Test organism	Inhibitory strain													
		X11	X13	X31	X40	X74	W42	650/1	653/1	651/10	WS/1	295/20	L324/1	701/2	795/2
Broth	1P5	1/10	1/160	1/320	1/320	1/320	1/80	–	–	1/40	–	1/80	1/80	–	1/10
	144F	1/20	1/320	1/320	1/320	1/320	1/80	1/160	1/80	1/160	1/160	1/320	1/320	1/320	1/320
Milk	1P5	–	1/40	1/160	1/160	1/160	1/40	–	–	–	–	–	–	–	–
	144F	–	1/160	1/160	1/320	1/320	1/80	–	–	1/20	1/10	1/80	1/40	1/10	1/20

Key: [Tables 1–6: – =not inhibited in lowest dilution, i.e. 1/10. . =no test.]

(2) *Activity test*

Milk cultures of the inhibitory strains were heat treated and stored in the refrigerator as for the dilution test. Whey from these heated cultures was then tested for power to inhibit acid production of starter cultures by modifying the activity test as used to detect the presence of bacteriophage (9).

From one of four bottles containing 25 ml. of sterile milk, 2·5 ml. of milk were withdrawn (bottle 4). To the milk in each bottle 1 ml. of an 18 hr. test culture (milk) diluted one part in three parts of milk was added. Whey from the heated cultures was added to the milk in bottles 2, 3 and 4 respectively and the test made as follows:

Bottle 1 (control) contained 1% milk culture of test organism only.

Bottle 2 contained test organism with 0·1% (i.e. 0·25 ml. of 1/10 dilution) of heated whey added.

Bottle 3 contained test organism with 1% (i.e. 0·25 ml.) of heated whey added.

Bottle 4 contained test organism with 10% (i.e. 2·5 ml.) of heated whey added.

After shaking, the cultures were incubated at 30° C. in a water-bath and inverted hourly. When the control culture had coagulated (after 5–5½ hr.) 10 ml. was removed from each bottle and titrated with *N*/10 NaOH. If the test cultures to which whey had been added gave a lower titration figure than the control, further 10 ml. samples were titrated after a total incubation period of 24 hr.

When, due to the addition of heated whey, inhibition of acid production by the test culture occurred, the extent of inhibition was calculated from the titration figures. The initial acidity of the sterile milk (taken as equivalent to 2 ml. *N*/10 NaOH) was deducted and the result expressed as a percentage (Tables 2, 3, 4 and 6). Where acid production by the test organism was entirely arrested the percentage inhibition, in theory, should be 100. In practice, the acidity of the added whey itself gave an initial increase in acidity in the sample under test compared with the control, and on titration after 5–6 hr. incubation lowered the inhibition percentage by 10–20. Hence where complete inhibition of acid production by the test organism occurred, the final percentage was between 80 and 90. After incubation for 24 hr., the acidity of the control had increased and the effect of adding 10% of acid whey initially, now only lowered the inhibitory figure 10%, percentages of 90–100 therefore indicated complete inhibition. Additions of 0·1 or 1% whey did not significantly affect the initial acidity.

(3) *Heat treatment of cultures before examination for inhibitory substance*

Nearly all the strains examined were able to produce acid rapidly in milk since they either originated from starters or were isolated with the object of using them as starters. When, therefore, the inhibitory effect of these cultures on the acid production of starter

Table 2. *Concentration of inhibitory substance obtained from a selection of inhibitory strains of lactic streptococci. Test organism—strain* 144 F

	Dilution test				Activity test					
Incubation period (hr.)	16		24		5			24		
	Test medium		Test medium		% whey added			% whey added		
Inhibitory streptococci	Broth	Milk	Broth	Milk	0·1	1	10	0·1	1	10
Starter strains:										
295/20	1/80	–	1/10	–	.	0	58	.	.	13
319/192/2	1/80	–	–	–	.	9	66	.	.	15
L324/1	1/160	1/20	1/160	1/10	.	35	85	.	11	24
252/2	1/80	–	1/10	–	.	9	48	.	.	.
252/8	1/320	1/20	1/160	1/10	.	21	68	.	7	19
251/6	1/320	1/20	1/80	–	.	16	68	.	.	12
252/5	1/320	1/20	1/160	1/10	.	18	68	.	12	19
650/5	1/320	1/20	1/320	1/10	.	14	62	.	0	21
651/5	1/20	–	–	–	.	0	18	.	.	.
652/5	1/320	1/40	1/320	1/10	.	13	65	.	.	15
R653/1	1/320	–	1/40	–	.	18	63	.	.	22
701/2	1/320	1/10	1/160	–	.	0	59	.	.	13
769/13	1/320	1/160	1/160	1/10	.	62	78	.	5	11
795/6	1/320	1/10	1/160	–	.	19	69	.	.	14
WS/1	1/320	1/10	1/160	–	.	5	68	.	.	.
'Wild' strains:										
N13	1/80	1/40	1/80	1/10	.	9	78	.	0	37
W42	1/20	1/40	1/10	–	.	23	73	.	11	35
X2	1/80	1/80	1/80	1/80	.	36	78	.	6	55
X15	1/640	1/320	1/640	1/320	94	84	86	21	92	93
X31	1/320	1/640	1/160	1/640	66	94	86	8	92	93
X43	1/640	1/1280	1/320	1/1280	90	86	84	60	95	93
X62	1/160	1/640	1/160	1/640	93	90	81	38	93	91
X72	1/160	1/640	1/160	1/320	100	93	76	19	96	92

Table 3. *Effect of heat treatment on the inhibitory substance. Test organism—strain* 144 F

				Activity test					
		Dilution test in		5–6 hr. % inhibitory whey			24 hr. % inhibitory whey		
Inhibitory strain	Heat treatment	Broth	Milk	0·1	1	10	0·1	1	10
W42	65° C. for 30 min.	1/40	1/80	0	68	81	.	4	86
	100° C. for 5 min.	1/80	1/80	0	67	78	.	7	88
X15	65° C. for 30 min.	1/320	1/320	55	84	74	10	93	86
	100° C. for 5 min.	1/320	1/320	48	87	77	11	88	86
X43	65° C. for 30 min.	1/320	1/640	91	86	86	3	84	81
	100° C. for 5 min.	1/640	1/640	95	95	84	13	90	84
795/6	65° C. for 30 min.	1/320	1/10	0	0	64	.	.	13
	100° C. for 5 min.	1/320	1/10	0	0	69	.	.	22

strains was being considered, it was important first to destroy the inhibitory organisms themselves after growth in milk. A simple method of killing the streptococci was to hold the tubes containing 24 hr. cultures in boiling water, but if this heat treatment should reduce the potency of the inhibitory substance it might be necessary to modify it. Hence the effect of placing tubes of culture in (1) boiling water for 5 min. and (2) a water-bath

at 65° C. for 30 min. was compared. Four inhibitory strains were incubated in milk at 22° C. for 24 hr. and the cultures divided into two portions which were heat treated as above and then rapidly cooled. The potency of the inhibitory substance in the whey of each pair of samples was compared (Table 3). There appeared to be no significant differences in the strength of inhibitory substances in the wheys after the different heat treatments and the former method which was the simplest for practical purposes was adopted.

Table 4. *Concentration of inhibitory substance produced in milk after different incubation periods at* 22° C. *Test organism—strain* 144 F

Inhibitory strain	Incubation period (hr.)	Dilution test in		Activity test: 5–6 hr. % inhibitory whey			Activity test: 24 hr. % inhibitory whey		
		Broth	Milk	0·1	1	10	0·1	1	10
X15	9	1/80	1/40	.	.	.	.	.	.
	24	1/640	1/1280	.	.	.	.	.	.
	48	1/320	1/1280	.	.	.	.	.	.
X71	8	1/20	1/10	0	0	87	0	0	15
	10½	1/160	1/160	0	90	95	0	15	98
	14½	1/320	1/320	71	81	87	48	98	98
	18½	1/320	1/320	100	100	95	8	98	93
	24	1/640	1/320	100	100	95	8	98	96
	48	1/640	1/640	0	96	87	.	6	90
X40	10½	1/40	1/80	0	41	87	.	6	90
	16½	1/160	1/320	70	87	82	8	87	87
	20	1/320	1/320	77	93	90	10	94	87
	26	1/320	1/640	84	85	83	91	91	90
	31½	1/320	1/640	90	89	87	96	93	90
	47	1/320	1/320	90	84	74	94	94	89
	70	1/320	1/320	91	91	79	58	92	87
	96	1/320	1/320	66	82	70	0	88	87

Table 5. *Comparison of* Str. agalactiae *with two strains of* Str. cremoris *as test organism*

Test organism	Inhibitory strain: W42	N13	251/5	251/6	251/8	251/10	251/11	251/12	X12	X15	X40	X43
Str. agalactiae	1/40	1/20	–	–	–	–	–	–	1/20	1/40	1/80	1/10
1 P 5	1/160	–	–	–	–	–	–	–	1/80	1/80	1/160	1/160
144 F	1/80	1/80	.	.	1/80	1/160	1/80	1/80	1/80	.	.	1/80

(4) *Period of incubation of cultures before examination of inhibitory power*

To determine the period of incubation which would yield whey with the highest inhibitory concentration milk was inoculated as usual with cultures of three inhibitory strains and incubated at 22° C., 10 ml. samples of the cultures were withdrawn after incubation periods of from 8 to 96 hr., heat treated and tested (Table 4).

(5) *Selection of test organism*

Hirsch (8) found *Str. agalactiae* to be a useful indicator strain against which to standardize preparations of inhibitory substance derived from a lactic streptococcus, but a test organism from within the actively acid-producing strains of lactic streptococci was desirable. Two *Str. cremoris* strains, 1 P 5 and 144 F, were compared with *Str. agalactiae* for use as test organism in the dilution test. The series in which *Str. agalactiae* was used as test organism was incubated at 37° C., the remainder at 30° C. The two *Str. cremoris*

Table 6. *Variation between strains of lactic streptococci in susceptibility to inhibitory substance. Percentage inhibition in activity test (5–6 hr.)*

Table 6. *Variation between strains of lactic streptococci in susceptibility to inhibitory substance.*
Percentage inhibition in activity test (5–6 *hr.*)

Inhibitory strain	% Added whey	*Str. cremoris* 144 F	1 P 5	318 B/27	299/1	267/13	M 6/10	L 236/28	725/33	W 15	W 40	*Str. lactis* M 1 S 1	N 31	N 40	X 13	X 31	X 43	X 71	M 2/4	M 16 S 1	N 13	T 2	W 42	W 35	W 6	X 14	X 38	X 40	X 62
Str. cremoris																													
251/5	1	0	.	.	.	.	.	.	.	0	12	0	0	0	0	0	.	0	0	21	.	0	0	0	12	0	0	.	0
	10	41	.	.	.	.	.	.	.	0	25	0	0	20	0	0	.	0	0	21	.	0	0	35	31	0	0	.	0
651/11	1	0	0	0	0	0	0	0	0	.	.	.	.	.	.	.	0	.	.	.	0	.	.	.	.	.	.	0	.
	10	64	0	0	0	0	0	0	63	.	.	.	.	.	.	.	0	.	.	.	0	.	.	.	.	.	.	0	.
654/3	1	0	.	.	.	.	.	.	.	0	0	0	0	0	0	0	.	0	0	0	.	0	0	0	26	0	0	.	0
	10	61	.	.	.	.	.	.	.	17	11	16	0	0	0	0	.	0	25	31	.	0	0	50	55	0	24	.	0
769/13	1	49	.	.	.	.	.	.	.	23	55	77	21	39	0	0	.	44	19	23	.	0	13	64	53	34	0	.	52
	10	72	.	.	.	.	.	.	.	66	45	56	29	48	0	0	.	40	65	74	.	0	35	64	80	62	75	.	52
795/6	1	20	0	0	0	0	0	0	10	0	18	43	0	27	0	0	0	26	45	15	0	0	8	16	27	0	0	0	0
	10	71	0	0	0	0	0	0	72	25	36	63	30	38	0	0	0	26	52	30	0	0	12	58	27	0	0	0	30
650/4	1	32	.	.	.	.	.	.	.	0	0	27	0	0	0	0	.	0	10	0	.	0	0	14	0	0	0	.	0
	10	68	.	.	.	.	.	.	.	10	20	34	0	0	0	0	.	0	43	50	.	0	0	43	64	0	36	.	0
Str. lactis																													
X 13	1	98	.	.	.	.	.	.	.	90	.	48	0	0	.	0	.	0	0	82	0	0	0	58	70	40	84	.	0
	10	90	.	.	.	.	.	.	.	90	.	80	15	0	.	0	.	0	86	85	29	73	86	92	78	88	85	.	0
X 15	1	100	90	75	56	93	78	40	87	.	.	.	.	.	.	0	.	.	.	.	0	.	.	.	.	.	.	0	.
	10	70	90	85	87	91	90	90	88	.	.	.	.	.	.	0	.	.	.	.	0	.	.	.	.	.	.	0	.
X 43	1	91	100	62	57	89	80	83	90	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	0	.
	10	82	82	76	82	78	90	83	82	.	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	0	.
X 71	1	100	87	40	46	90	62	53	.	87	93	10	.	0	0	0	0	.	0	50	0	0	77	50	93	37	87	0	0
	10	85	80	40	85	80	75	80	.	77	82	51	.	0	0	0	0	.	64	81	0	37	83	70	80	83	81	0	0
N 13	1	0	0	21	7	15	42	13	90	0	0	0	18	0	18	0	27	0	16	12	.	0	0	0	0	0	0	0	.
	10	80	10	85	53	15	70	68	79	0	30	67	48	73	57	15	70	24	92	83	.	0	86	85	74	59	0	40	.
W 42	1	87	88	37	0	88	74	20	92	.	.	.	.	.	.	.	0	.	.	.	0	.	.	.	.	.	.	.	.
	10	83	82	74	71	80	74	67	82	.	.	.	.	.	.	.	0	.	.	.	0	.	.	.	.	.	.	.	.
X 40	1	100	93	64	74	90	83	87	.	.	.	.	.	.	.	.	0	.	.	.	0	.	.	.	.	.	.	.	.
	10	81	87	78	77	88	80	80	.	.	.	.	.	.	.	.	0	.	.	.	0	.	.	.	.	.	.	.	.
X 62	1	88	.	.	.	.	.	.	.	96	.	0	0	0	0	0	.	0	46	66	0	0	26	50	93	50	90	.	.
	10	77	.	.	.	.	.	.	.	86	.	38	0	0	0	0	.	0	65	83	0	0	58	75	80	77	79	.	.

strains proved to be more sensitive to the inhibitory substance than *Str. agalactiae* and the use of the latter was discontinued. Of the two *Str. cremoris* strains 144 F appeared to be the more sensitive (Tables 1 and 5). Strain 144 F was, therefore, chosen as the test organism against which the effect of all the inhibitory strains was compared.

(6) *Selection of growth medium and its influence on concentration of inhibitory substance produced*

The strains were originally selected on the basis of ability to produce inhibitory substance when grown in Y.D.B., but it was possible that growth in milk might yield different concentrations of inhibitory substance. Comparisons were made of the concentrations produced, using seventeen strains, after growth in Y.D.B. and milk respectively and, with the exception of a few strains which failed to coagulate milk on incubation of a 1% inoculum for 24 hr. at 22° C., higher concentrations of inhibitory substance were obtained after growth in milk. In all further tests the inhibitory substance was produced during growth in milk rather than in Y.D.B., since this would also give a better indication of the effect these strains might have on cheese starter strains. After growth in milk the concentration of inhibitory substance was assayed in the whey only, no attempt being made to determine the concentration in the curd.

(7) *Examination of the test organism* (144 F) *recovered after action of inhibitory substance*

A comparison of the results obtained after incubation for 16 and 24 hr. in the dilution test (Table 2) and after 5 and 24 hr. in the activity test (Tables 3 and 4) often showed a recovery in growth of the test organism where the added inhibitory whey was in high dilution. The following procedure was adopted to investigate whether the organism which grew after a longer incubation period was a variant, resistant to the inhibitory action.

Tubes from the dilution test, originally seeded with the test strain 144 F and containing dilutions of whey from the inhibitory strains X 15 or X 43, were selected which showed no growth after incubation for 24 hr. but in which the broth became turbid and the milk coagulated after the incubation was increased to 42 hr. (The control tests usually showed obvious growth after 5–7 hr.) The chosen cultures were plated on yeast-dextrose agar and 24 colonies picked into litmus milk. The rate of acid production in milk varied from culture to culture, but this was not regarded as significant since it is a normal feature when single-strain starters are plated. These cultures were retested by the dilution and activity tests using the respective original inhibitory wheys.

(8) *Preparation of mixed cultures of inhibitory and non-inhibitory strains of lactic streptococci*

Starter strains for which phages had previously been isolated but which were not inhibitory to other strains were selected. The non-inhibitory strains were combined in pairs with inhibitory strains, so that the latter were not attacked by the phages which lysed the former. The cultures, after incubation at 22° C. for 18 hr. in milk, were mixed so that the mixtures of the two strains contained the following percentages of inhibitory strain: 100, 75, 50, 25, 10, 1, 0·1, 0·01, 0·001 and 0. One per cent inoculum of each mixture was added to 10 ml. quantities of sterile milk, incubated at 22° C. and subcultured daily. A modified activity test was applied to the mixture after incubation for 18 hr. and 4 days

using phage to show any alteration in the acid production attributable to the non-inhibitory strain in the mixture; this method of employing phage has been detailed elsewhere (10). To two bottles containing 25 ml. of milk a 1% inoculum of the mixture was added, and 0·1 ml. of phage suspension in Y.D.B. corresponding to the non-inhibitory strain was added to one bottle and 0·1 ml. sterile Y.D.B. to the other for control purposes. A comparison of titration figures (typical set, Table 7) obtained after incubation at 30° C. for 5–6 hr. indicated to what extent the non-inhibitory strain had contributed to the acid production of the mixture.

III. RESULTS AND DISCUSSION

(1) *Factors affecting extent of inhibitory action*

(i) *Difference between strains in ability to produce inhibition*

Of the 276 strains of lactic streptococci initially examined, 78 strains produced, during growth in culture media a substance which inhibited the development of other lactic streptococci.

From 56 'wild' strains isolated from sour milk for use as starter strains, 19 (34%) were found to be inhibitory. Although strains from many different samples of milk were examined, these strains arose from eight samples of milk and it is likely, therefore, that some strains may have been identical. Fifty-nine (27%) inhibitory strains from 220 'cultivated' strains of *Str. cremoris* were obtained out of a large collection of starters; several of these inhibitory strains may also have been similar since they were obtained from only 13 starters.

All the inhibitory strains from starter were *Str. cremoris* whilst those from 'wild' sources were classified as *Str. lactis*, although several of the latter gave atypical reactions to some differential tests (11).

There seemed to be no outstanding characteristic by which strains producing the inhibitory substance could be distinguished from other strains of these streptococci. Other workers (3, 5, 6) have also noted that the strains of lactic streptococci which had been isolated from milk strongly inhibited similar strains of lactic streptococci.

(ii) *Variation in concentration of inhibitory substance produced by different strains*

The activity and dilution tests were used to compare the concentrations of inhibitory substance produced by different strains and a representative selection of the results is given in Table 2.

The 'wild' strains were outstandingly inhibitory compared with strains of *Str. cremoris* isolated from starter. Within the 'wild' strains, X15 inhibited in dilutions of 1/640 and X43 inhibited in 1/1280 and were examples of the strongest inhibitory strains encountered, whilst N13 and W42 had a less marked effect on the test organism.

(iii) *Variations in susceptibility of lactic streptococci to the inhibitory principle*

Twenty-eight strains of lactic streptococci, 10 *Str. cremoris* and 18 *Str. lactis* were used as test organisms in the activity test and the wide range of inhibitory effects of 14 different strains on these test organisms is recorded in Table 6. Of all the test organisms examined a strain of *Str. cremoris*, 144F, was the most susceptible to the inhibitory substances produced by the 14 strains tested. Not only was it inhibited by the whey from all the

inhibitory streptococci, but also to a greater extent than the other strains. Other susceptible strains of *Str. cremoris* were 725/33, 267/13 and W40. Amongst the strains of *Str. lactis* W6, W35 and M16S1 were readily inhibited, whilst the growth of strains X13, 31, 40 and 43, N13 and T2 was not affected by the whey from the majority of the inhibitory streptococci. No strain resistant to inhibition by all these inhibitory streptococci was encountered.

(iv) *Heat treatment of inhibitory cultures*

As indicated earlier (p. 401) there was no reduction in potency of the inhibitory substance when heated in whey at 100° C. for 5 min. as compared with a heat treatment of 65° C. for 30 min. Whitehead (3) has previously indicated that steaming for 30 min. reduced the potency of the inhibitory substance compared with pasteurization at 63° C. for the same time. Oxford (6) stated that the inhibitory substance, diplococcin, which he obtained from similar streptococci was heat stable at pH 4·0 but slowly inactivated at pH 6–7 when held in a boiling water-bath. As the cultures in milk when heat treated were acid in reaction (approx. pH 4·5) heat stability of the inhibitory principle was to be expected and probably Whitehead's results were due to the fact that the culture he tested only developed an acidity of 0·16% (lactic acid) before heat treatment.

(v) *Period of incubation of inhibitory strain*

The results in general show (Table 4) that the greatest concentration of inhibitory substance was present after incubation for 1 day. The potency of the inhibitory substance increased in the whey of X71 for incubation periods up to 24 hr. Strain X40 after incubation for 26 hr. gave the greatest strength of inhibitory substance and when the incubation period was extended to 4 days, there was no further increase. The inhibitory principle appears to have been produced during the active growth of the organism and it will be seen also that with prolonged incubation the potency of the inhibitory substance diminished slightly.

(2) *Nature of inhibitory action*

(i) *Effect of inhibitory substance on test organism* (144F) *recovered after inhibition*

Cultures which at first appeared to have been inhibited grew after further incubation. Strains were taken from this delayed growth in one instance, and were found to be inhibited to the same degree as the original organism (144F) and appeared to be identical with it.

In phage action when strains appear after extended incubation, they remain resistant to the phage applied, and thus differ from those strains which appear similarly when a strain has been subjected to the action of inhibitory organisms.

(ii) *Effect of mixing inhibitory with non-inhibitory strains*

It was found that, when the inhibitory strain formed more than 10% of the original mixture of inhibitory and non-inhibitory strains, after daily subculture for 4 days, the non-inhibitory strain ceased to contribute to the acid production of the mixture as shown by the addition of phage in the activity test. In some mixtures the presence of only 1% of the inhibitory strain was sufficient to ensure its predominance within 4 days.

When a non-inhibitory strain (e.g. strain 144F) was very susceptible to the inhibitory

substance, the inhibitory strain was predominant after 1 day, even when it had formed only 1% of the original mixture.

The results indicate that when the constituent strains of a starter mixture are mixed in approximately equal proportions if one strain possesses inhibitory properties, it may quickly predominate. Whilst the inhibitory strain is gaining predominance the activity (ability to produce acid rapidly in milk) of the starter may be reduced. This temporary loss of activity is demonstrated by figures given in Table 7. After incubation for 18 hr.

Table 7. *Effect of mixing inhibitory strain* 251/10 *with non-inhibitory strain* 144 F

Mixture no.	Percentage of each strain in original mixture		Activity test. Time after mixing			
			18 hr.		4 days	
	Inhibitory strain 251/10	Non-inhibitory strain 144 F	Control	Phage 144*f* added	Control	Phage 144*f* added
1	100	0·0	5·3	5·0	5·3	5·4
2	75	25	4·1	4·4	5·5	5·4
3	50	50	4·7	4·6	5·0	5·7
4	25	75	4·7	4·8	5·4	5·8
5	10	90	4·6	4·5	5·1	5·4
6	1	99	4·1	3·5	5·3	5·7
7	0·1	99·9	6·0	2·0	4·9	4·2
8	0·01	99·9	5·9	2·0	6·7	2·3
9	0·001	99·9	6·1	2·0	6·3	2·2
10	0·0	100	5·9	2·0	6·9	2·0

The titration figure given is the no. of ml. of *N*/10 NaOH required to neutralize the acidity of 10 ml. of inoculated and incubated milk. Titration figure for inoculated milk before incubation = 2·0 ml. *N*/10 NaOH.

mixture no. 6 gave a titration figure of only 4·1 compared with 5·3 for strain 251/10 alone and 5·9 for strain 144 F alone. A comparison of the control titration figure (4·1) with that of the sample to which phage 144*f* has been added (3·5) shows that both strains were contributing to acid production at this stage. After 4 days' incubation addition of phage 144*f* did not reduce the titration figure in the activity test and it may be assumed that the inhibitory strain had then become entirely responsible for acid production and the titration figure (5·3) was the same as that for strain 251/10 alone (no. 1).

Milk is known to be a source of inhibitory streptococci (4, 5) and it is perhaps not surprising that inhibitory strains should be encountered during the search for starter strains. Hence it is necessary to test any new strain, suitable in other characteristics for starter, for production of inhibitory substance in milk before use as a starter strain. If this test is not made before a new strain is introduced as starter and several strains are prepared separately and finally mixed as they are added to the cheese vat, one strain may inhibit the others and the beginning of cheesemaking will be 'slow'. Eventually the cheesemaking will proceed normally (provided no other factor interferes, i.e. phage) as the inhibitory strain itself multiplies, increases in activity and compensates for the loss of activity by the other strains in the starter.

The presence of inhibitory strains in starters themselves, i.e. amongst commercial strains of *Str. cremoris*, does not seem to have been noted before. It appears that 'slowness' may result from mixing several commercial starters at the time of making cheese even though the inhibitory action of strains derived from starter appears to be milder than that of inhibitory strains isolated from milk.

SUMMARY

1. Inhibitory strains of lactic streptococci have been encountered amongst strains of *Str. cremoris* isolated from starter and amongst strains of *Str. lactis*, isolated from sour milk, which had otherwise been regarded as suitable for cheesemaking.

2. Methods are described for determining the concentration of inhibitory substance produced and the effect of these inhibitory strains on a wide selection of lactic streptococci.

3. The significance of the presence of strains of lactic streptococci having inhibitory properties, if incorporated in starters, particularly when the strains are prepared separately and mixed in the vat, is discussed. Recommendations for the exclusion of inhibitory strains from such starter mixtures are made.

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374. THE IDENTITY OF STREPTOCOCCI FROM STARTER AND OF STREPTOCOCCI, SUITABLE FOR USE AS STARTER, ISOLATED FROM SOUR MILK

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The failure of cheese starter, due to attack by bacteriophage, is a phenomenon familiar to workers concerned with the cultivation and selection of streptococci suitable for use in cheese manufacture. It may be said that almost all strains of lactic streptococci used as starter are attacked by phage in the factory sooner or later but it is apparent that some strains may not be so liable to phage attack as others, whilst other strains again may not be attacked so completely.

Numerous strains of lactic streptococci have been accumulated in the course of other work during the seasons 1942–6, and it was thought that, if the identity of these strains were established, useful clues to the susceptibility of the different strains to lysis by phage, which might assist also with the selection of strains for starter, might emerge.

The strains examined were grouped as follows:

(i) 277 '*cultivated*' *strains from commercial starters consisting of:*

(*a*) 137 strains for which phages causing lysis had been obtained. Both the strains and the corresponding phages were isolated and purified (1) from samples of starter and whey from cheesemaking factories during the course of investigations into starter 'slowness'. Some of the starters came from Continental sources, New Zealand and from the U.S.A.

(*b*) 116 strains for which no phage had been isolated at the time of testing. At first, the only method of obtaining the corresponding phage for a particular strain of the lactic streptococci was to introduce it as a starter into a cheese factory, where phage attack was normally heavy and to await the appearance of phage in the whey. It has now been found possible to build up phages capable of lysing strains, for which no phage had previously been isolated, by 'feeding' whey samples from a cheese factory or bulk-milk samples, after acidification and filtration, to the respective strains.

Phages have also been obtained for some strains which did not react with test dilutions of other lytic filtrates, by addition of concentrated undiluted phage suspension capable of lysing some other strain of the lactic streptococci. The method was adapted from that used by Wilson (2) for building up phages for previously untypable strains of staphylococci. If any lysis appeared when 'neat' phage was applied to a previously unattacked strain growing on agar, the new phage was propagated on that strain. Recently, by applying the last two methods, phages have been isolated for the majority of the strains in this group.

(*c*) 24 sub-strains isolated from platings of commercial starter strains. Phages had not been isolated for these strains.

(ii) 72 *'wild' strains originating from sour milk*

These were milk strains obtained during the search for lactic streptococci which could be used for starter. Altogether 129 milk samples of poor bacteriological quality, from different farms, were allowed to sour, usually at 30° C. but occasionally at 22 or 37° C. When a sample had coagulated, if the clot was reasonably free from gas holes and from digestion, it was plated on yeast-dextrose agar.* Occasionally thallium acetate (1:2000) was added to the milk before incubation or to yeast-dextrose agar on which the milk samples, after coagulation, were streaked. Thallium acetate inhibits many common organisms, but has less effect on the growth of streptococci. Colonies were picked after two days' incubation at 30° C., inoculated into litmus milk and incubated at 30° C. Most of the cultures which were picked reduced litmus and formed an acid clot. These strains were selected for further study. They were subcultured daily in milk for about a week using a 1% inoculum and incubated at 22° C. If at the end of this period of subculturing, the milk culture coagulated after incubation at 22° C. for 24 hr., a milk activity test (1) was carried out on the culture. When the acidity produced by the culture during an activity test merited the inclusion of the strain in a starter, the culture was retained. About 10% of the strains although suitable for starter up to this point were discarded after the activity test, since during growth in milk they developed an unpleasant malty smell and flavour. Hunter (3) has also reported a high proportion (25%) of strains obtained from similar sour-milk sources which produced a caramel or malty taint. 653 strains were picked, but only 72, from 34 different samples, were retained. In the search for 'wild' strains of lactic streptococci suitable for starter, attempts were also made to isolate strains from silage using media containing 1:2000 thallium acetate. Silage was found not to be a good source of strains of lactic streptococci for starter. The few which were isolated grew only slowly at 30° C. and coagulated milk after several days.

Methods of differentiating cultures

The tests used for the differentiation of the cultures of lactic streptococci were based on the work of Sherman (4) and his co-workers. Before this investigation was completed, Hunter (3) published work on similar lines and recent work by Abd-El-Malek & Gibson (5) also became available. The present work has been reconsidered in the light of the differential tests used by these workers.

In litmus milk culture all the strains isolated from starters rapidly reduced the litmus, before acid coagulation, and fresh isolations from sour milk were selected by this characteristic so that three species of the enterococcus division (4), namely, *Str. faecalis*, *Str. liquefaciens* and *Str. zymogenes* were the only streptococci with which the lactic streptococci might be confused. Hence the cultural and biochemical tests used were mainly employed in differentiating streptococci of the enterococcus division from the lactic and in differentiating *Str. cremoris* from *Str. lactis*. Table 1 shows the reactions of these organisms to the differential tests, which are given in detail below, considered to be typical by Sherman (4) and Hunter (3).

The inoculum of the strains used throughout the tests, unless stated, consisted of two drops (1/10 ml.) of an actively growing 18 hr. culture grown in yeast-dextrose broth. This inoculum was added to 10 ml. of dextrose-lemco broth.†

* Peptone 2%, lemco 1%, dextrose 0·5%, salt 0·5%, Yeastrel 0·3% and agar 2%.

† Peptone 1%, lemco 1%, dextrose 1% and salt 0·5%.

(1) *Heat resistance, survival after holding at* 60° C. *for* 30 *min.*

The tubes, after inoculation of the broth, were immersed in a water-bath (thermostatically controlled to approx. ±0·25° C.) at 60° C. and held for 30 min. They were then cooled and incubated at 30° C. and the presence or absence of growth was recorded after 24 hr.

(2) *Growth at* 40° *or* 45° C.

After inoculation the tubes were incubated in a water-bath at 40 or 45° C. for 24 hr. after which they were examined for growth. Tests were not always done at 45° C. since, if it were established that no growth occurred during incubation at 40° C. no additional information would thereby be obtained. The test medium used originally was dextrose-lemco broth, but the test at 40° C. was repeated in litmus milk, since Abd-El-Malek & Gibson (5) found litmus milk to be a better differential medium.

(3) *Inhibition of growth by* 4% *sodium chloride*

Four per cent chloride was added to the broth and after inoculation the tubes were incubated at 30° C. for 24 hr. A few tests were made with some of the strains in broth containing 6·5% salt.

(4) *Ability to tolerate* pH 9·2 *or* 9·6

The broth was adjusted to the desired pH immediately before inoculation, and the tubes were then placed in an anaerobic jar with soda-lime and incubated at 30° C. for 24 hr. Uninoculated control tubes were also incubated and the pH was checked when the readings were made.

(5) *Growth in litmus milk*

After inoculation into litmus milk the cultures were incubated at 30° C. and examined at short intervals for reduction. The formation of an acid clot within 24 hr. was recorded as positive. Other cultures, although showing reduction and acidity, were recorded negative. At this stage a microscopical examination was made.

(6) *Activity test*

Many of the strains were examined for activity (1) when first isolated. Other strains were tested after they had been in the cold store for several months; consequently, even though subcultured daily for a week before testing, the activity might have been higher when they were first isolated. The strains were grouped into two classes, (*a*) high-acidity producers—suitable for starter (+), (*b*) low-acidity producers—unsuitable for starter (−).

(7) *Fermentation tests*

Fermentation tests were made in 1% peptone water containing 0·5% salt, 0·01% Andrade's indicator and 0·5% of the test 'sugar', solutions of which had been sterilized by 'Seitz' filtration and added to the heat-sterilized culture medium. Five ml. quantities of the media were inoculated with a loopful of a 24 hr. yeast-dextrose broth culture, incubated at 30° C. and acid reactions recorded after 5 days. The reactions in five 'sugars' were tested, viz. sucrose, mannitol, raffinose, maltose and dextrin, selected for the following reasons:

Sucrose. Orla-Jensen (6) has recently suggested that saccharose fermenting lactic streptococci occurring in milk products might be called *Str. saccharolactis* without finally

deciding at this stage whether they are related to *Str. lactis* or to the other saccharose fermenting streptococci.

Mannitol. Often fermented by *Str. faecalis*, not fermented by *Str. cremoris* and may or may not be fermented by *Str. lactis*. Its fermentation is helpful in classifying these strains.

Raffinose. Rarely fermented by *Str. cremoris* and *Str. lactis*.

Maltose and dextrin. According to Hunter(3) fermented by *Str. lactis* but not by *Str. cremoris*.

(8) *Hydrolysis of arginine*

The method of Niven, Smiley & Sherman(7) was used. The medium contained 0·5% tryptone, 0·2% K_2HPO_4, 0·05% glucose, 0·3% l(+) arginine mono-hydrochloride and 0·25% Yeastrel which replaced 0·5% yeast extract, a modification suggested by Abd-El-Malek & Gibson(5). Ten ml. quantities of this broth were inoculated with one drop of a 24 hr. test culture in yeast-dextrose broth and incubated at 30° C. for 2 days. The production of ammonia was detected by addition of one drop of the growth medium to one drop of Nessler's reagent on a spot plate.

The results of these tests are presented in Table 2.

Table 1. *Typical reactions of streptococci to tests employed for their differentiation based on:*

(*a*) Sherman's classification(4)

	Survival 60° C. 30 min.	Growth at 40° C.	Growth at 45° C.	Growth in presence of 4% salt	Growth in presence of 6½% salt	Growth in presence of pH 9·2	Growth in presence of pH 9·6	Litmus milk: Reduced before coagulated	Litmus milk: Reduced acid coagulated after 24 hr.	Morphology. Diplococci and/or streptococci
Enterococcus:										
Str. faecalis	+	+	+	+	+	+	+	+	+	+
Str. liquefaciens										
Str. zymogenes										
Lactic:										
Str. lactis	+	+	−	+	−	+	−	+	+	+
Str. cremoris	±	−	−	−	−	−	−	+	+	+

(*b*) Hunter's differentiation(3)

	Growth at 10° C.	Growth at 40° C.	Growth at 45° C.	Growth and acid production at 37° C.	Acid produced from Maltose	Acid produced from Dextrin	Morphology in milk at 37° C. after 4–6 hr.
Lactic:							
Str. lactis	+	+	−	Rapid	+	+	Diplococci and short chains
Str. cremoris	+	−	−	Marked inhibition	−	−	Streptococci and long chains

Results and discussion

(1) From a comparison of Tables 1 and 2 it appears that all the 'cultivated' strains obtained from commercial starters exhibited the characteristics of *Str. cremoris*. The results of the tests are remarkably uniform considering the large numbers of strains examined (277) and the wide range of source from which they were obtained. The agreement with Sherman's classification for *Str. cremoris* is very close, the only variation being that none of the strains survived heating at 60° C. for 30 min. Shattock & Mattick(8) report that *Str. lactis* does not usually survive this treatment, and it appears that under the conditions of the test *Str. cremoris*, also, fails to survive. Maltose and dextrin, too, were very useful for differentiating *Str. cremoris* from *Str. lactis*. The *Str. cremoris* strains

Table 2. *Characteristics of 'cultivated' and 'wild' lactic streptococci*

Reaction	Survival 60° C. 30 min.	Growth in dextrose-lemco broth at 40° C.	Growth in dextrose-lemco broth at 45° C.	Growth in presence of 4% salt	Growth in presence of 6½% salt	Growth in presence of pH 9·2	Growth in presence of pH 9·6	Litmus milk: Reduced before coagulated	Litmus milk: Reduced acid coagulated 24 hr.	Morphology. Diplococci and/or streptococci	Activity 6 hr. 30° C.	Acid produced from: Sucrose	Mannitol	Raffinose	Maltose	Dextrin	Growth in litmus milk at 40° C.	Ammonia produced from arginine
(1) 'CULTIVATED' STRAINS FROM COMMERCIAL STARTERS (277)																		
+	0	0	0	2	0	0	0	277	259	277	73	0	0	0	13	1	0	0
+?	0	0	0	10	0	0	0					3	0	1	4	0	0	4
–	277	277	144	265	0	277	277	0	18	0	16	253	256	254	237	255	228	224
(2) 'WILD' STRAINS FROM NATURALLY SOURING MILK (72)																		
+	0	24	0	70	0	14	0	72	72	72	72	29	42	0	68	59	69	70
+?	0	18	0	2	0	4	0					0	2	0	0	0	0	0
–	72	30	72	0	13	54	72	0	0	0	0	42	27	71	3	12	2	1

did not ferment dextrin and only a small percentage of those examined fermented maltose. Sucrose was not attacked.

Yawger & Sherman (9) report that out of a total of 25 strains of *Str. lactis* which they used for differential tests, 19 had been isolated from starter and it appears that *Str. lactis* in starters then used in U.S.A. may have been more common. Whitehead & Hunter (10) believe that cheese starters consist mainly of strains of *Str. cremoris* and Knudsen (11), too, considered that most of the starter organisms are varieties of *Str. cremoris*. Even so, it is surprising to find that of the large number of strains examined from a wide range of starters in commercial use, not one was *Str. lactis*. Both *Str. cremoris* and *Str. lactis* sour milk rapidly and since, in cheesemaking the main function of a starter strain is speedy and steady acid production, it is reasonable to expect that some strains of *Str. lactis* might be isolated. If the streptococci obtained from starters which have been reported here, had consisted of mixtures of *Str. lactis* and *Str. cremoris*, even with the latter predominant, it is possible that one might have been less susceptible than the other to phage lysis. Information on the identity of strains within the division of lactic streptococci might have proved valuable for the selection of strains for starter, but, as all the strains isolated were *Str. cremoris*, further guidance in choosing suitable strains for starter, cannot be expected along these lines.

(2) The identification of the 'wild' strains, selected as suitable for starter on the basis of acid production when grown in milk, was less definite, using the classifications of Sherman and Hunter, than that of the 'cultivated' strains. The 'wild' strains are clearly lactic streptococci and there was no possibility, using the proper tests, of confusing the strains isolated with *Str. faecalis*. It will be seen that 75% of the strains failed to grow in broth at pH 9·2 showing, in this respect according to Sherman, a similarity to *Str. cremoris*, but 97% grew in 4% salt and 33% grew at 40° C. in dextrose-lemco broth, exhibiting the tolerance to these tests typical of *Str. lactis*. The failure of all the lactic streptococci to survive 60° C. for 30 min. has already been noted.

On ability to ferment maltose and dextrin, a large number of 'wild' strains were classified as *Str. lactis*, although a few did not ferment both. Many of these strains fermented mannitol as well as maltose or dextrin and since mannitol is not attacked by *Str. cremoris* it seems to indicate that many 'wild' strains were *Str. lactis*.

It was difficult on the results of these tests to group all the 'wild' strains either as *Str. lactis* or *Str. cremoris*, since many appeared to be 'intermediates'. Some workers do not differentiate between *Str. lactis* and *Str. cremoris*. Babel (12) considers the whole group of lactic streptococci as *Str. lactis*. If they were to be divided into two species, only two of these 'wild' strains (N 31 and N 40) could possibly be *Str. cremoris*, although some exhibited one or more of the characteristics typical of *Str. cremoris* given in Table 1. Allowing the same latitude in the interpretation of results 65% were *Str. lactis* and the remainder 'intermediates' or more probably variants of *Str. lactis*.

Abd-El-Malek & Gibson (5) state that the identification of organisms of the *Str. lactis-Str. cremoris* group presented no difficulties. 'In every instance those that formed ammonia from arginine produced obvious changes in litmus milk at 40° C. and were identified as *Str. lactis*; those that did not form ammonia failed to show growth at 40° C. and were classified as *Str. cremoris*.' By applying these two tests to most of the cultures originally examined, it has been possible to classify with two exceptions all the 'intermediates' discussed above as *Str. lactis*; N 31 and N 40 have been classified as doubtful *Str. cremoris*.

It has been noticed that in other respects, e.g. phaging ability and inhibitory power (13), the 'wild' strains resemble one another more closely than they resemble the 'cultivated' strains. It is not surprising to find, using arginine and growth in litmus milk at 40° C., that the 'wild' strains fall into the same group.

Str. cremoris was detected by Abd-El-Malek & Gibson in ten raw milk samples, but amongst the 'wild' strains selected from a large number of raw-milk samples recorded here, *Str. cremoris* was rarely found. Other surveys of 'wild' lactic streptococci not discussed here have shown similar results and the rarity of *Str. cremoris* strains is possibly due to the different treatment of the milk samples before plating. From their susceptibility to lysis by phages of other strains N 31 and N 40 might be grouped as *Str. lactis*, but it has not been possible, using the methods already outlined (3, 4, 5), to identify them definitely as either *Str. cremoris* or *Str. lactis*.

(3) It is evident that three tests: action in litmus milk, hydrolysis of arginine and ability to grow in litmus milk at 40° C. (5) will usually be sufficient to differentiate clearly between *Str. lactis* and *Str. cremoris*. Four from 228 'cultivated' strains of *Str. cremoris* produced slight ammonia from arginine and three were from the same source. One 'wild' strain (N 31) appeared in all respects to be *Str. cremoris* except that it produced ammonia from arginine. Growth in litmus milk, instead of broth, at 40° C. was more decisive where results had been doubtful. Of 48 'wild' strains which showed slight growth or failed to grow in broth at 40° C., 46 grew in litmus milk. In the isolation of strains suitable for starter, these three tests will normally be sufficient to identify *Str. lactis* and *Str. cremoris*, but the possibility of isolating strains of *Str. faecalis*, reducing litmus and rapidly forming an acid clot in milk which may be confused with *Str. lactis*, cannot be ignored. It has been emphasized by Abd-El-Malek & Gibson (5) that the number of *Str. faecalis* strains, vigorously reducing litmus, which they isolated from milk was less than expected. In the collection under discussion, no strains of *Str. faecalis* were found. If they were originally present, they were eliminated because of slow acid production.

The fermentation of dextrin might also be useful for differentiation since it was fermented by most strains of *Str. lactis* but by only two strains of *Str. cremoris*.

(4) Of the 'wild' strains 41 % fermented sucrose. The results show that the evidence is insufficient to create a species *Str. saccharolactis*, suggested by Orla-Jensen (6).

SUMMARY

1. 277 strains of lactic streptococci isolated from commercial starters and 72 'wild' strains from souring milk, suitable for cheesemaking, have been identified. All the starter strains examined were *Str. cremoris* and those (with two possible exceptions) from sour milk were *Str. lactis*.

2. Since the strains from each source were so similar, the information obtained did not help in the selection of the strains, within each group, most suitable for starter.

3. It is suggested that three differential tests are sufficient to distinguish *Str. lactis* and *Str. cremoris* and to identify them when isolating strains suitable for starter.

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